AD_____

Award Number: DAMD17-00-1-0043

TITLE: Development of a Biologic Basis for PSMA Targeting in Prostate Cancer

PRINCIPAL INVESTIGATOR: Warren D. W. Heston, Ph.D.

CONTRACTING ORGANIZATION: Lerner Research Institute
Cleveland Clinic Foundation
Cleveland, Ohio 44195

REPORT DATE: February 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2001	Annual (1 Feb 00 - 31 Jan 01)

4. TITLE AND SUBTITLE
Development of a Biologic Basis for PSMA Targeting in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0043

6. AUTHOR(S)
Warren D. W. Heston, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lerner Research Institute
Cleveland Clinic Foundation
Cleveland, Ohio 44195

E-Mail: hestonw@ccf.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

We are going to define the biology of PSMA in order to enhance our ability to use PSMA as a target and to generate anti-tumor agents that will be specific and selective for PSMA protein. There is a PSMA-like protein that is similar to PSMA. We will identify that protein so we can develop agents that will be selective for the prostate PSMA protein. We want to know whether the binding of drugs that bind to PSMA are transported into the tumor or whether they stay at the cell surface as it will alter the strategy for drug development. We are going to develop potential ligands (drugs or molecules that bind to PSMA) that will serve as a guide to the chemicals that bind to the active site of enzymatic activity of the PSMA, the "substrate pocket" to identify the 3D structure of the PSMA. The identification of the 3D structure of the PSMA will greatly enhance the potential for rapid development of selective agents. We will also use phage display to identify small peptide ligands that could service as drug delivery vehicles and to aid to identify ligands that will differentiate between PSMA and PSMA-like protein.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate Cancer, PSMA Targeting	39
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

PSMA is a strongly expressed protein in prostate cancer, and it is a type-two membrane protein with the bulk of the molecule and enzymatic activity outside of the cell and thus is accessible to therapeutic attack. We discovered that PSMA has activity as a unique folate hydrolase, being a glutamate carboxypeptidase. We also observed that cells expressing PSMA were sensitive to killing by polygammaglutamated methotrexate while non-PSMA expressing cells were resistant. We therefore proposed to explore the biologic potential of PSMA as a therapeutic target in terms of transport and prodrug activation. Because PSMA appeared to be a broadly active carboxypeptidase we proposed to examine polygammaglutamated derivatives of cytotoxics as potential agents that then might be transported into the cell or as prodrugs that would be activated by the enzymatic activity of PSMA releasing the drug from the polygammaglutamated-linked cytotoxic at the site of the tumor.

BODY

Aim #1 of 2: Characterize the structural, enzymatic , and transport activity of PSMA and PSMA like proteins for rational development of biology based targeting strategies. Our statement of work Task 1 for this aim in the first year:

a. **Clone enzymatically active PSMA and PSM'-like protein for secretion from CHO, bacterial, and/or baculoviral cells to generate the amounts required for x-ray diffraction.**

   We have cloned PSMA into a baculoviral expression system, however the yield of purified material has not been large enough or pure enough for the proposed x-ray diffraction studies. The recombinant material has not been enzymatically active, which eliminates rationale of using analysis of the results of x-ray studies of the enzymatic active site to predict the design requirements for generation for more active inhibitors and substrates. We will continue our attempts to accomplish this in the second year because it is necessary to accomplish this goal first prior to beginning (b). We may have to change to using CHO cells or to genetically modify the PSMA protein to make it a secreted protein.

b. **Begin x-ray characterization of the active site- second year.**

   Refer to "a."

c. **Characterize the binding, enzymatic activity, transport, and toxicity of PSMA and PSMA-like proteins with iodomethotrexate, methotrexate, polygammaglutamates and N-acetylaspartylglutamate.**

Our hypothesis was that PSMA serves as a binding/transport protein. Within this hypothesis, the polygammaglutamated form itself is transported into an acidic endosome and allows for the polygammaglutamated chemical species to assume the nondisassociated unionized form. It is thus able to diffuse out of the endosome and into the cell cytoplasm. Once in the cell at pH 7.4 the glutamates change to the ionized form and become trapped in the cytoplasm, in essence a transport system of sorts. We considered that substrates could enhance the internalization rate. PSMA does appear to have a spontaneous rate of internalization and antibodies can further induce internalization. A major disappointment in our experimental findings has been the observation that ligands (such as polygammaglutamated methotrexate) and inhibitors (PMPA, suramin, and quisqualate) do not increase internalization. The results of our experiments have been contrary to our hypothesis. We reasoned that some proteins need to be cross linked to induce internalization. The polygammaglutamated substrates were chemically modified to be bivalent in case cross-linking of PSMA molecules was required for internalization. Again, none of these experiments demonstrated increased internalization. It did not matter whether the glutamate substrates were alpha-linked glutamate substrates such as N-acetylaspartylglutamate. Indeed none of the substrates for the hydrolytic enzymatic activity of PSMA induced internalization.

A number of antibodies to PSMA have been further explored. Not all antibodies that bind PSMA will induce internalization of PSMA. Thus there is likely a region of the PSMA protein that is responsible for internalization "transport" that is different from the enzymatic hydrolytic domain of PSMA.

Even so, there is a spontaneous slow endocytosis of PSMA that occurs without antibody binding. This process could be considered involved in "transport". Methotrexate gamma-triglutamate does kill PSMA expressing cells, and does not kill non-PSMA expressing cells. This is considered because PSMA hydrolyzes the triglutamate, freeing MTX which then used the folate transporter to gain entrance to the cell. Methotrexate-triglutamate's activity as a toxin is decreased by inhibitors such as PMPA, consistent with blocking binding to the enzyme. This would also decrease any potential endosomal transport.

To further examine the transport aspect, this coming year we will be using agents which would effect endosomal pH. This will allow binding but not allow a pH drop in the endosome. Theoretically, this would prevent the transport of the polygammaglutamated agents for agents that are being taken up in this manner.

That neither polygammaglutamated nor alpha linked glutamated peptides serve to induce internalization is one of the reasons funding was requested to identify this as a biologic parameter for further drug targeting. The lack of tranport means that PSMA can not be viewed as another class of folate

binding protein nor transport protein. It narrows the spectrum for antitumor targeting in PSMA expressing cells. PSMA can still be seen as a target wherein the PSMA expression removes the glutamate and then allows for the activity going from an inactive agent to an active agent in a prodrug fashion, but reduces the possibilities for a "drug vector approach" based on increased uptake and retention relative to transport and retention of any proposed polygammaglutamated targeting agent.

Because of these findings and the delay in achieving active recombinant protein for the x-ray diffraction studies, our second year focus is directed to achieving purification of active PSMA for x-ray diffraction studies and for enzymatic characterization. PSMA enzymatic activity may still be useful for defining enzymatic means to generate selective and active pro-drugs and thus our focus in the second year will be to further identify potential pro-toxic substrates for PSMA.

Aim #2 of 2: The second specific aim included the generation of a number of polygammaglutamated analogues.

PSMA appears to be a broad-spectrum carboxypeptidase with broad activity in hydrolytically removing glutamates. However we have synthesized and examined the polygammaglutamated derivative of the multi-targeted folate antagonist LY231514. LY231514 is a modern antifolate. Polygammaglutamated LY231514 was toxic to cells but exhibited no differences in cytotoxicity between PSMA expressing and non-expressing cells. This raises concerns even about using PSMA as a Pro-Drug strategy. Still it appeared that methotrexate triglutamate was differentially active on PSMA non-expressing and expressing cells and we will investigate at least two other drugs for prodrug activity prior to any decision to not pursue this strategy. We will cut back on this aim and only synthesize two additional polygammaglutamated molecules to provide further characterization of whether the enzymatic range of specificity of PSMA is as broad was we initially thought.

New Directions:

Because the strategy is still evolving it may be possible to use the hydrolytic domain as a target for other strategies such as redirecting gene therapy. We are currently studying a number of gene therapy strategies based on promoter enhancer specificity to drive prodrug-activating enzymes. We will investigate whether we can use polygammaglutamated linkages to direct gene vectors developed in other work to PSMA expressing cells.

We have identified the promoter and enhancer for PSMA in other studies and we will determine whether we can develop that strategy in a synergistic strategy with the prodrug strategy being developed here.

KEY RESEARCH ACCOMPLISHMENTS

- Defined enzymatic substrates as not responsible for inducing internalization of PSMA.
- Defined LY231514-Glu 3 as not differentially cytotoxic to PSMA expressing relative to PSMA non-expressing cells.

REPORTABLE OUTCOMES

Abstracts

1. Uchida A., O'Keefe DS., Bacich DJ., Watt F., Molloy PL., Heston WDW.: Prostate-specific suicide gene therapy using the newly discovered prostate-specific membrane antigen (PSMA) enhancer. Proc. Amer. Assoc. Cancer Res. 42:691, 2001. (Abst#3717)

2. Grasso YZ., Heston WDW.: Prostate-specific membrane antigen as a target for prodrug therapy: relationship between its folate hydrolase and endocytosis activities. Proc. Amer. Assoc. Cancer Res. 42:777, 2001. (Abst# 4169)

3. O'Keefe DS, Uchida A, Bacich DJ, Watt FB, Martorana A, Molloy PL, Heston WDW.: Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. Prostate 45: 1490157, 2000.

CONCLUSIONS

The hydrolytic domain of the enzymatic activity of PSMA is different from the domain associated with inducing internalization. Thus, targeting strategies will have to take these differences into account. Potential drugs that are substrates are unlikely to induce internalization and will have to act like prodrugs; that is to be toxic following the enzymatic release of glutamate from the glutamylated prodrug cytotoxic agent.

REFERENCES

I. Uchida A., O'Keefe DS., Bacich DJ., Watt F., Molloy PL., Heston WDW.: Prostate-specific suicide gene therapy using the newly discovered prostate-specific membrane antigen (PSMA) enhancer. Proc. Amer. Assoc. Cancer Res. 42:691, 2001. (Abst#3717)

II. Grasso YZ., Heston WDW.: Prostate-specific membrane antigen as a target for prodrug therapy: relationship between its folate hydrolase and endocytosis activities. Proc. Amer. Assoc. Cancer Res. 42:777, 2001. (Abst# 4169)

III. O'Keefe DS, Uchida A, Bacich DJ, Watt FB, Martorana A, Molloy PL, Heston WDW.: Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. Prostate 45: 1490157, 2000.

IV. O'Keefe DS, Bacich DJ, Heston WDW.: Prostate specific membrane antigen. In Prostate Cancer: Biology, Genetics, and the New Therapeutics (Eds LWK Chung, WB Isaacs, JW Simons) Humana Press Inc Totowa NJ, pp 307-326, 2001.

APPENDICES

Hard copies of the references have been supplied.

From: Atsushi Uchida, M.D.
To: Warren D. Heston, Ph.D.
Date: 5/2/01 1:07PM
Subject: Re: Meetings and abstracts

Skip

I was too late to read your groupwise mail.

There is an abstract for AACR 2000.

Prostate-Specific Suicide Gene Therapy Using the newly discovered prostate-specific membrane antigen (PSMA) Enhancer.

Atsushi Uchida, Denise S. O'Keefe, Dean J. Bacich, Fujiko Watt, Peter L. Molloy, and Warren D.W. Heston.

Departments of Cancer Biology and Urology, The Cleveland Clinic Foundation, Cleveland, OH 44195; and CSIRO Division of Molecular Science, North Ryde, NSW 2113, Australia.

Abstract

PSMA is expressed by both benign and malignant prostatic epithelium and upregulated by androgen deprivation. Therefore, PSMA is an attractive therapeutic target for advanced prostate cancer. Recently, the enhancer driving prostate-specific expression of the PSMA gene was cloned by Watt *et al.* (unpublished data). We then subcloned a number of enhancer-deletion constructs and tested them for maximum activity. We subsequently cloned the most active region of the enhancer into a plasmid containing the PSMA promoter driving expression of the suicide gene cytosine deaminase (CD) from *E. coli*. The specificity of this technique was examined *in vitro* using C4-2 (the highly metastatic subline of LNCaP cells), a breast cancer (MCF-7), a colon cancer (HCT-8), and a lung cancer (H-157) cell line. The cells were transiently transfected and the IC50 of 5-fluorcytosine (5-FC) and 5-fluorouracil (5-FU) of the cells was determined by the MTS assay. Toxicity of 5-FC on each transfected cell line was compared to that with parental cell line. The IC50 of 5-FU varied among cell lines (0.5-10uM), whereas that of 5-FC was similar (>10 mM). Enhanced toxicity of 5-FC in tranfected C4-2 cells was shown down to an IC50 of 300 uM in a dose dependent manner. MCF-7, HCT-8, and H-157 cells, which do not express PSMA, were not significantly sensitized by transfection. Transfection efficiency was similar among the cell lines (C4-2; 8%, MCF-7; 15%, HCT-8; 6%, H157; 10%). A significant cytotoxicity was shown specifically in PSMA expressing cells, due to a bystander effect.

This work was supported in part by a grant from the A.F.U.D./A.U.A. Research Scholar Program & the C.R. Bard Foundation (D.S.O'K.) & grant PC990017 from the U.S. Army (D.S.O'K.), & NIH grant DK/CA47650 (W.D.W.H.).

AACR 2001 Online Abstract Submitter
92nd Annual Meeting
March 24-28, 2001
New Orleans, LA

Abstract ID: 958

Instructions

Tips and Frequently Asked Questions

Submission Guidelines

Abstract Checklist

Authors

Title

Body

Keywords/Indexing

Abstract Category and Subclassification

Sponsor

Awards/Support for Attendance

Copyright Transfer

Payment Information

Other Options

Presenting Author Contact Information

Logout

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AS A TARGET FOR PRODRUG THERAPY: RELATIONSHIP BETWEEN ITS FOLATE HYDROLASE AND ENDOCYTOSIS ACTIVITIES

Grasso, Ying Z, Heston, Warren D
The Cleveland Clinic Foundation, Cleveland OH

Prostate-specific membrane protein (PSMA), a type 2 transmembrane glycoprotein, is highly expressed in malignant prostate epithelia and in the tumor-associated neovasculature of several solid tumors. PSMA has folate hydrolase activity when methotrexate tri-gamma-glutamate was used as a substrate. PSMA has also been shown to endocytose more rapidly when stimulated by monoclonal antibodies against its extracellular domain. However, little is known about the relationship between the regions on PSMA which are responsible for antibody binding and for its hydrolase activity. In the present study, we investigated these issues by using substrates and high affinity antagonists for PSMA's hydrolytic activity and several monoclonal antibodies against different regions in PSMA's extracellular domain. Our results showed that none of the substrates and antagonists could induce PSMA endocytosis in C4-2 cells, a LNCaP cell line derivative. On the other hand, several monoclonal antibodies induced PSMA internalization while a few others did not. We also performed competition experiments to study whether the PSMA domains for hydrolysis and antibody binding overlap. Interestingly, the antibodies which induced PSMA internalization competed with J591, an antibody known to cause PSMA endocytosis, for binding and uptake, while the substrates and the antibodies that did not induce PSMA internalization failed to compete with J591. Therefore, our data suggested that different domains on PSMA are responsible for antibody binding and for its folate hydrolase activity. Continued investigation is needed to further determine the relationship between these two domains and the implication in prodrug delivery using PSMA as the membrane target. [This work is supported in part by a grant from U.S. Army Medical Research Acquisition Activity, DAMD 17-00-1-0043 (WDWH).]

18

Prostate Specific Membrane Antigen

Denise S. O'Keefe, PhD, Dean J. Bacich, PhD, and Warren D. W. Heston, PhD

1. INTRODUCTION

The molecular basis of prostate carcinoma has always been less understood than that of other cancers, despite its high incidence in the population. One of the reasons for this is that the molecular pathways leading to prostate cancer do not seem to parallel that of other cancers, and until recently there have been few markers for this tumor. One of the most exciting recent findings in prostate cancer was the discovery of prostate specific membrane antigen (PSMA). PSMA is a glutamate carboxypeptidase that switches from a cytosolically located protein in the normal prostate to a membrane-bound protein in prostatic carcinoma. The majority of PSMA expression appears to be restricted to the prostate, with some expression seen in the brain, salivary glands, and small intestine. Intriguingly, our group recently found that PSMA is expressed in the endothelial cells of the neovasculature of nearly all solid tumors examined. The membrane-bound nature of this protein—and the limited sites of expression as well as expression in tumor-associated neovasculature—makes PSMA an ideal marker and therapeutic target for clinical studies and treatment of not only prostate cancer, but of other solid tumors as we progress into the 21st century.

2. THE DISCOVERY OF PSMA

The antigen itself was discovered by Horoszewicz et al. *(28)*, who isolated LNCaP cell membranes and immunized mice with the mixture, producing the antibody known as 7E11C5.3. The LNCaP cell line is derived from a Lymph Node metastasis from a Carcinoma of the Prostate, and is considered the most relevant of the few prostatic cell lines available, because it retains expression of prostate specific antigen (PSA), prostatic acid phosphatase (PAP) and the androgen receptor, among other characteristics typical of human prostate cancers in vivo *(23,25,29,30)*. Characterization of the antibody revealed that it specifically bound epithelial cells of normal prostate, benign prostatic hypertrophy (BPH), and prostatic carcinoma specimens, making PSMA an attractive prostate-specific marker *(28)*. The rights to the 7E11C5.3 antibody were then bought by a biotechnology company called Cytogen Corporation (Princeton, NJ).

Cytogen modified the antibody so that it could be labeled with ^{111}Indium while retaining its specificity, and renamed it Cyt-356. The radiolabeled antibody was then

From: *Prostate Cancer: Biology, Genetics, and the New Therapeutics*
Edited by: L. W. K. Chung, W. B. Isaacs, and J. W. Simons

administered to nude mice carrying tumors established from the LNCaP cell line. After 3 d, 30% of the injected dose had localized to the LNCaP xenograft, with no significant amounts found in other tissues *(42)*. This occurred despite the fact that the same investigators had noted that immunohistochemical staining using this modified antibody against normal human tissues showed weak reactivity with cardiac muscle, proximal kidney tubules, and sweat glands. There was also strong binding to a subset of skeletal muscle cells. A phase I clinical study using radiolabeled Cyt-356 as an imaging agent for metastatic deposits was then carried out in patients with prostate cancer and known distant metastases *(78)*. No adverse affects of the agent were noted in the patients, and the results showed promise for use of the immunoconjugate as an imaging agent. At this stage it was clear that a deeper understanding of the molecular basis and function of PSMA expression in prostate cancer was needed.

3. CLONING AND EXPRESSION PATTERN OF PSMA

The complementary DNA (cDNA) sequence encoding PSMA was cloned in 1993 using a classic textbook approach *(33)*. The monoclonal antibody Cyt-356 was used to immunoprecipitate PSMA from LNCaP cell membranes, and the protein was then electrophoresed on and isolated from a polyacrylamide gel. PSMA was then subjected to proteolytic digestion and the subsequent peptide fragments were microsequenced to determine their amino acid composition. Based on the amino acid sequence, degenerate oligonucleotide primers were designed that could theoretically amplify the PSMA cDNA sequence from LNCaP reverse-transcribed mRNA. The resulting PCR product was cloned and used to probe a LNCaP cDNA library and isolate the full-length PSMA transcript of 2653 nucleotides (the sequence can be found in Genbank under the accession number M99487).

Translation of the cDNA sequence predicted that the protein consists of 750 amino acids with a molecular wt of 84 kDa before posttranslational modifications *(33)*. It was later shown that in vitro translation of the PSMA cDNA sequence with and without dog pancreatic microsomal membranes (which permit glycosylation of proteins to occur in vitro) produces proteins of 100 and 84 kDa, respectively. This is consistent with the 100 kDa molecular wt of PSMA seen in LNCaP cells *(32)*. PSMA is a Type II integral membrane protein, and as such the short N-terminal of the protein is located on the cytoplasmic side of the membrane, with the majority of the protein located on the extracellular side of the membrane, making it available for clinical and therapeutic targeting *(33)*. Northern analyses using the PSMA cDNA probe and ribonuclease protection assays using a probe corresponding to nucleotides 242–588 of the PSMA cDNA sequence showed no expression of PSMA mRNA in the prostatic cell lines PC-3 and DU145, and no expression in normal tissues from kidney, liver, lung, mammary gland, pancreas, placenta, skeletal muscle, spleen, and testis. However, there was high expression in normal prostate and prostatic carcinomas, and barely detectable expression in salivary gland, whole brain, and small intestine. Expression of PSMA mRNA in BPH specimens was either reduced relative to that of normal prostate, or absent altogether *(32)*, which is most likely an indication of the major cell type that constitues BPH (stromal cells that do not express PSMA), rather than a biologic phenomenon. Interestingly, our group and others recently found that PSMA is expressed in the

endothelial cells of neovasculature associated with almost all solid tumors, but not in normal vasculature *(40,61)*. Immunohistochemistry using five different antibodies against PSMA has confirmed this, as have *in situ* hybridization and RT-PCR results *(8,9)*. In fact, the only tumor which does not seem to consistently express PSMA in the associated vasculature is that of the prostate (2/12 prostate cancer specimens expressed PSMA in the vasculature), perhaps providing a clue to the function of PSMA in these cells *(9)*.

As PSA expression is modulated by androgens, Israeli et al. examined the effect of various steroids on PSMA expression in LNCaP cells *(32)*. In contrast to PSA expression, PSMA is downregulated in the presence of androgens, with the highest amount of PSMA expressed in LNCaP cells grown in charcoal-stripped (and therefore steroid-reduced) media. This finding was later supported both in vitro in LNCaP cells, and in vivo by Wright et al., *(76)*, who found that in 55% (11 of 20) and 100% (4 of 4) primary and metastatic tumor specimens, PSMA expression was significantly upregulated in patients who had undergone some form of hormonal deprivation, relative to matched specimens from the patients before treatment. These findings are particularly significant, because of the implication that PSMA can be a highly useful clinical and therapeutic target for patients with recurrent disease.

4. REGULATION OF PSMA EXPRESSION: CLONING OF THE PSMA PROMOTER AND ENHANCER

To obtain more information about the genetic regulation of PSMA expression, we set out to determine the complete sequence of the gene. A bacteriophage P1 library containing fragments of DNA from normal human lymphocytes approx 60–80 kb in size was screened using PCR. Two sets of oligonucleotide primers were used—one set corresponding to the 5′ end of the PSMA cDNA sequence, and one set corresponding to the 3′ end of the sequence. The advantage of this method of screening was that the gene spanned more than 60 kb of DNA, and two P1 clones that overlapped by about 5.6 kb had to be analyzed to acquire the entire sequence *(50)*.

Comparison of the genomic and cDNA sequences of the PSMA gene revealed 19 exons ranging in size from 64 to 379 nucleotides, and 18 introns from 300 to 7363 base pairs (Fig. 1). The entire genomic sequence of the gene can be found in Genbank, under accession number AF007544. One of the most striking features of the genomic sequence was the presence of a CpG island at the 5′ end of the gene. From nucelotides 2661–2990 of the genomic sequence—which extends from exon 1 into the first intron of the gene—the observed/expected ratio of the CpG dinucleotide was 1.85, which is significantly greater than the ratio for bulk human DNA (0.25)*(1,50)*. CpG islands are substrates for DNA methyltransferase, and the presence of a CpG island in the 5′ region of the PSMA gene suggests a role for DNA methylation in the regulation of PSMA expression.

Once we had the genomic sequence of the 5′ portion of the gene, we were able to clone the promoter controlling transcription of PSMA mRNA. To confirm the transcription start site indicated by the initial PSMA cDNA sequence *(33)*, we carried out 5′ Rapid Amplification of cDNA Ends (5′ RACE). 5′ RACE is a form of PCR that uses one primer based in the known cDNA sequence of the gene, and one primer that binds to 5′ ends of all mRNA transcripts. Thus, only one primer is specific to the gene of

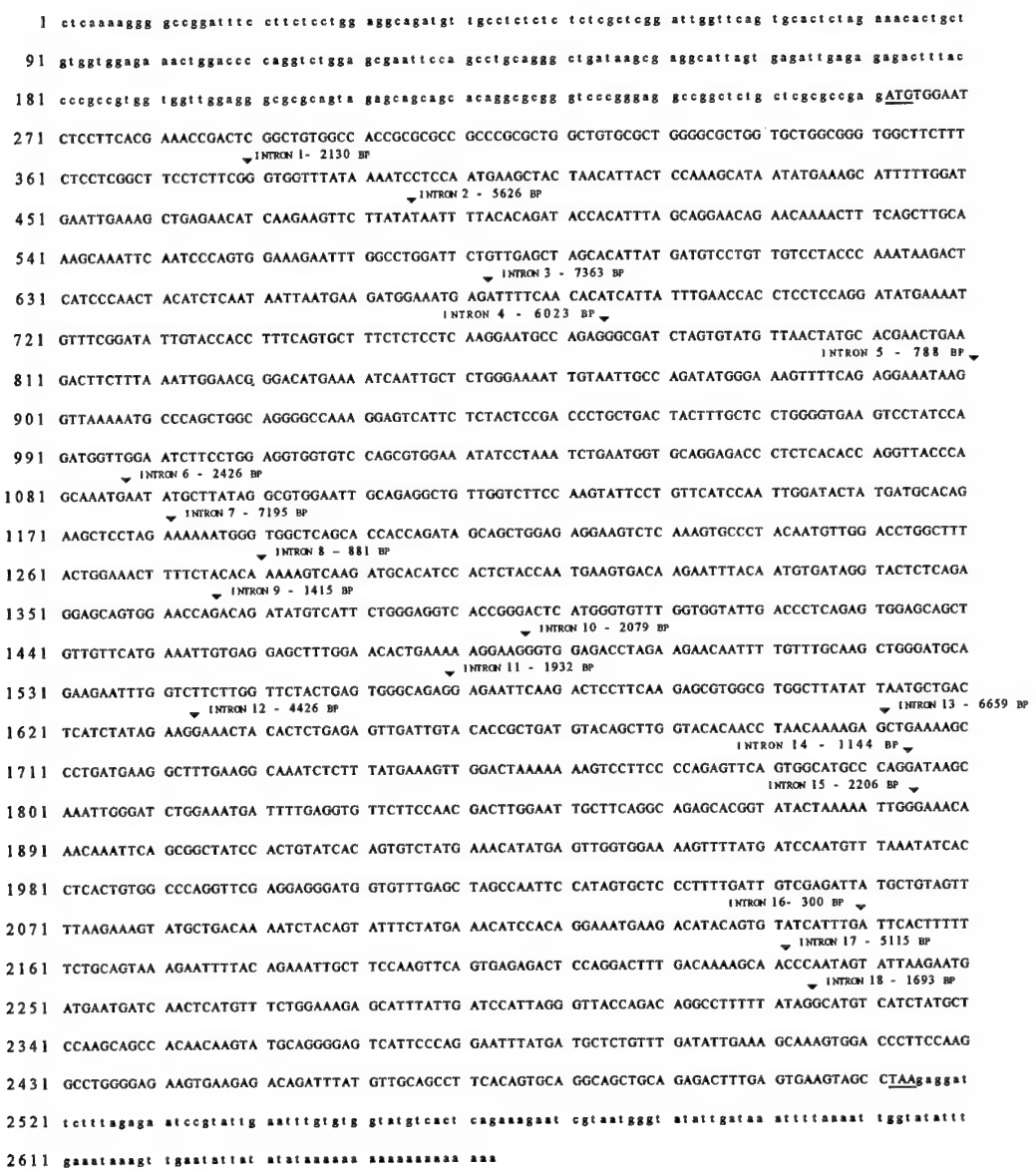

Fig. 1. The cDNA sequence of the human PSMA gene. The 5′ and 3′ untranslated regions are shown in lower case; the coding region is in upper case. The start and stop codons are underlined, and intron positions and sizes are indicated by the arrowhead.

interest, but by using this method it is possible to determine the exact start sites of transcription and therefore predict the sequence encompassing the promoter region of the gene. Our 5′ RACE experiments confirmed the original start site (+1 of the cDNA sequence), but also showed other start sites within this region, at –195 and –235, in addition to the original start site at –262 relative to the translation initiation codon of the gene. Such heterogeneity of transcription start sites is not uncommon in genes where the promoter lacks a TATA box. Consistent with this finding, the DNA sequence upstream of these start sites has no typical TATA boxes *(50)*. We next cloned the 1244

base pair region of genomic DNA spanning the start sites, and approx 1 kb of the region 5′, into a reporter vector to test whether the region was capable of driving transcription of the firefly luciferase gene. To our surprise, not only did this region of the gene have significant activity/mg of cell protein relative to the strong SV-40 viral promoter/enhancer in LNCaP cells, but it also appeared to be prostate specific, in that we could not detect significant levels of luciferase in the DU145 and MCF-7 cell lines (prostate and breast carcinoma lines). These cell lines do not express PSMA, and therefore presumably do not contain the appropriate trancription factors for activation of the PSMA promoter.

Interestingly, we did see luciferase expression driven by the PSMA promoter in PC-3 cells—another prostatic cell line that does not express PSMA—at levels corresponding to about 10% of that of the control SV-40 promoter/enhancer *(50)*. This led us to examine the genomic region of PC-3 DNA containing the CpG island for hypermethylation by Southern analysis with methylation sensitive restriction enzymes. We were able to demonstrate that while DNA from normal male lymphocytes and the LNCaP cell line was not hypermethylated, DNA from PC-3 cells was at least partially hypermethylated in this region. We next treated PC-3 cells with the demethylating agent 5-azacytidine, but were unable to detect expression of PSMA after treatment with the drug (O'Keefe et al., unpublished data), and we are still investigating this phenomenon.

In further experiments to delineate the minimal promoter region of the gene, we discovered that our original estimation of the strength of the PSMA promoter was somewhat high. We had calculated the strength of the PSMA promoter relative to that of the usually strong SV-40 promoter/enhancer in the luciferase-reporter experiments. However, when we transfected various cell lines with the SV-40 promoter alone—and after adjusting for transfection efficiency compared these transfections to those with the SV-40 promoter/enhancer combination—we were surprised to find that in LNCaP cells, addition of the SV-40 enhancer to the basal SV-40 promoter did not enhance reporter gene transcription and in some cases repressed it. In contrast, in other cell lines such as PC-3, the SV-40 promoter/enhancer combination significantly increases reporter gene transcription (O'Keefe et al., unpublished data). Other laboratories have reported similar findings in LNCaP cells when combining the SV-40 enhancer with other basal promoters such as the PSA minimal promoter (Peter Molloy, personal communication) although the reason for this remains unclear.

Deletion constructs of the promoter region allowed us to localize the minimal promoter to between bases 461–1097. The original promoter construct had contained an *Alu* repeat sequence, and we also found that once this region was deleted, reporter gene expression increased (Horiguchi et al., unpublished data). Although PSMA expression is regulated by androgens *(32,76)*, there are no typical androgen-response elements in the promoter region or in the entire PSMA genomic sequence. However, because there might be novel androgen response elements, we tested the deletion constructs and the original promoter construct for androgen responsiveness in LNCaP cells.

Although the minimal PSMA promoter described here appeared to exhibit prostate-specificity, it could only promote basal levels of reporter gene expression *(75)*. Watt et al. *(75)* cloned the PSMA enhancer region (PSME) using an "enhancer trap" system. The enhancer trap library was created by partial digestion of the P1 bacteriophage clones containing the PSMA genomic sequence, and subcloning the resultant fragments

into a vector containing the PSMA promoter-driving expression of the Green Fluorescent Protein (GFP) gene. Screening of the library for DNA fragments able to increase GFP expression over that seen by the promoter alone was carried out in LNCaP cells and a number of other non-PSMA expressing cell lines. Using this method, a fragment of DNA that was able to increase transcription from the PSMA promoter by 250-fold was identified. When the enhancer was linked to other stronger basal promoters instead of the PSMA promoter, transcription levels were increased by at least 10-fold; in the most impressive experiment, the PSME was linked to the herpes virus thymidine kinase (TK) promoter and transcription was nearly threefold that of the Rous-Sarcoma Virus promoter/enhancer, which in itself is a strong viral promoter. The PSME retains prostate-specificity even when linked to the TK promoter—which is not prostate restricted in expression—and the PSME also shows repression by androgens *(75)*. As such, the PSME shows excellent promise for use in gene therapy approaches targeting prostate cancer in the near future.

5. ALTERNATIVE SPLICING OF THE PSMA GENE

Using RT-PCR of normal prostate tissue, Su et al. *(64)* discovered the first reported mRNA splice-variant of the PSMA gene. The variant, PSM′ (PSM-prime), transcribes from the regular PSMA promoter and uses an alternative 5′ splice donor site within exon one of the gene, deleting bases 114–379, which includes the translation start codon for PSMA. Initiation of translation of the PSM′ protein begins at nucleotide 427, producing a glycoprotein of about 95 kDa that lacks the intracellular and transmembrane domains of PSMA. As such, PSM′ is located within the cytoplasm, but still retains the enzymatic activity of PSMA *(21)*. RNase protection assays differentiating PSMA from PSM′ mRNA transcripts revealed that in normal prostate PSM′ is the dominant isoform, whereas in prostate tumors and the LNCaP cell line PSMA is more prevalent *(36,64)*. Compilation of the data to form a "tumor index" comparing the ratio of PSMA : PSM′ resulted in a score of 9–11 for LNCaP cells, 3–6 for prostate carcinoma, 0.75–1.6 for BPH, and 0.075–0.45 for the normal prostate. Unfortunately, further analysis of the ratio of PSMA : PSM′ in clinical specimens has not been reported, so it remains unclear whether or not this tumor index could have a clinical impact.

Another alternative splice form of PSMA that was isolated from the human brain, prostate, and liver deletes amino acids 657–688 of the protein *(5)*, and creates an amino acid substitution (Asn→Lys). These amino acids correspond to the entire 18th exon of the gene, so the splicing event probably occurs by "exon skipping." We have also seen this splice form in cDNA derived from a colon tumor. Further investigation of this variant is required to determine whether it retains the activity of the full length PSMA protein, and whether it exists in significant levels relative to PSMA and PSM′.

Finally, when we implemented 5′ RACE of the PSMA gene using LNCaP cells, we discovered a number of novel transcripts. The first, which we have called PSM-C, begins transcription at the same nucleotides as the PSMA and PSM′ transcripts, then uses the same splice donor site as PSM′ (nt 114), but uses an alternative splice acceptor site located within intron one. Nucleotides 3270–3402 of the genomic PSMA sequence are transcribed, followed by exon 2 and exon 3. Translation of this variant containing a previously unidentified exon, which we have termed exon 1b, would result in a protein

identical to PSM′. Another variant, PSM-D, again uses the same splice donor site as PSM′, and a unique splice acceptor site in intron one, including another novel exon (exon 1c) which is from nucleotides 4289–4389 of the genomic PSMA sequence. The putative translation of this protein reveals a new translation-initiation start site located in exon 1c, followed by 42 novel amino acids and the rest of the PSMA protein in-frame. Interestingly, a motif in the novel region consisting of the peptide Ala-Ala-Tyr-Ala-Cys-Thr-Gly-Cys-Leu-Ala is similar to that seen in the growth-factor cys-knot family of proteins. Using RT-PCR, we were able to demonstrate the existence of this variant in normal prostate and LNCaP cells; however, we were unable to demonstrate significant amounts of this mRNA splice variant via RNase protection assays on these tissues.

At least one other group has also found splice variants arising from novel exons in intron one of the gene; one exon continuing on from the 3′ end of exon 1 for 68 nucleotides, and another extending for 97 nucleotides. All three variants include exon 1 (nt 1–379), and thus would be predicted to translate into a protein with a transmembrane domain. One of the variants contains both new exons aligned in tandem, and the three variants are expected to produce proteins between 40 and 805 amino acids in length *(53)*. Novel variants such as those described here have not been proven to contribute to or be functionally involved in prostate cancer. Therefore, their clinical significance remains uncertain.

6. MAPPING OF THE PSMA GENE AND IDENTIFICATION OF THE PSMA-LIKE GENE

Chromosomal localization of the PSMA gene has proven to be controversial. Initial mapping by two independent research groups using Fluorescent *In Situ* Hybridization (FISH) and the full-length cDNA sequence as a probe indicated two regions for the gene—11p11-12, and 11q14 *(38,56)*. To identify the true location of the PSMA gene, Leek et al. *(38)* used PCR of somatic cell hybrids containing various regions of chromosome 11, assigned the gene to 11p, and suggested that the 11q14 locus represented a PSMA pseudogene. Rinker-Schaeffer et al. used two P1 clones containing approx 120 kb of the PSMA gene and surrounding sequence to repeat the FISH experiment, and under conditions of high stringency, assigned the gene to 11q14 *(56)*. Later, it became apparent that FISH can be subject to artifact under conditions of high stringency when one of the regions involved is close to a centromere, which in this case is the 11p11 locus. We therefore mapped the gene again, using a number of sets of oligonucleotide primers designed to bind both intronic and exonic sequences of the gene, and PCR against a panel of somatic cell hybrids containing various regions of chromosome 11. We found that the PSMA gene does map to 11p11, approx 7 Mb from D11S1350. We also established that the "PSMA pseudogene" sequence reported on Genbank as mapping to 11q14 (accession number HSU93599) did not exist, but instead a gene that is highly homologous to the PSMA gene resides on 11q14.3 *(50)*. Further analysis of the gene at the 11q14 locus (which we have termed the "PSMA-like" gene, Genbank accession number AF261715), revealed that exons 2–19 of the PSMA gene are duplicated on the long arm of chromosome 11, along with their corresponding introns. We have been unable to detect duplication of the promoter region, or of exon

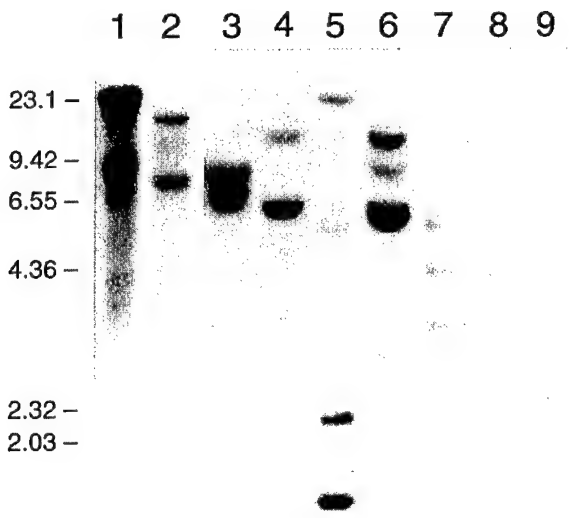

Fig. 2. Zoo-Blot using PSMA cDNA as a probe. Genes homologous to PSMA exist in many other species, including yeast. Lanes 1–9 are hybridized with genomic DNA from: (1) human, (2) monkey, (3) rat, (4) mouse, (5) dog, (6) bovine, (7) rabbit, (8) chicken, and (9) yeast.

one *(50)*. This would suggest a different mechanism of regulation, and therefore a different tissue expression pattern of the PSMA-like gene. The exonic sequences of the two genes are highly conserved (97% identical), and we have determined that the PSMA-like gene is transcribed and expressed in kidney and liver tissue, but not in prostate samples (O'Keefe et al., unpublished data). Complete characterization of the PSMA-like gene is needed. Thus, clinical and therapeutic strategies targeting PSMA can be designed to avoid PSMA-like expressing tissues or targets, and subsequently limit lack of specificity and unecessary toxicity.

7. MOLECULAR EVOLUTION OF THE PSMA FAMILY OF PROTEINS

Comparison of the intronic sequence differences of the PSMA and PSMA-like genes allowed us to calculate that evolutionary period in which the original gene duplicated was 22 million years ago *(50)*. This data is consistent with the report that the tyrosinase gene, which is closely linked to the PSMA-like gene on chromosome 11q14, was subject to duplication to 11p 24 million years ago *(17)*. It would therefore seem that the locus at 11q14 contained the original gene, and that a little more than 20 million years ago—after the divergence of man and rodent, but before the separation of man and chimp—this locus was duplicated on 11p11. Both genes then evolved further, with the PSMA gene gaining expression in the prostate. Southern blot analysis has shown that PSMA homologs exist in many species, and this is supported by the finding of homologs as far back in evolution as yeast and *C. Elegans* (Fig. 2; Heston et al., unpublished; *[55]*). Similarly, it is interesting to note that although PSMA homologs have been cloned in several of these species, there are no reports of PSMA expression in the prostate *(2,5,22)*. Instead, these PSMA homologs seem to be primarily expressed in the

Table 1
Homology Between Human PSMA and Selected Homologs and Paralogs

Species	Protein	Size (aa)	AA/NT homology to PSMA	Folate Hyd. activity	DPP IV activity	NAALA-Dase activity	GenBank accession number	Reference
Human	PSMA	750	100/100%	+	+	+	M99487	*(8)*
	NAALADase II	740	67/74%	nt	+	+	AJ012370	*(30)*
	NAALADase L	740	35/ns%	nt	+	–	AJ012371	*(30)*
	PGCP	542	27/ns%	nt	nt	+	AF119386	*(29)*
	DPP IV	766	29/ns%	nt	+	nt	M80536	*(33)*
	Transferin Receptor.	760	31/ns%	–	–	–	M11507	*(34)*
Mouse	MoPSM (PSMA homolog)	752	86/85%	+	nt	+	AF026380	*(2)*
Rat	NAALADase (PSMA homolog)	752	86/85%	+[a]	nt	+	U75973	*(21)*
	I100[b]	746	41/ns%	nt	+	–	AF009921	*(35)*
Pig	Folypoly-γ-glutamate Carboxypeptidase (PSMA homolog)	751	91/88%	+	nt	+	AF050502	*(27)*

[a] Bacich et al. unpublished observations;
[b] rat homolog of NAALADase L protein; The amino acid (AA) and nucleotide (NT) homologies to PSMA are shown; NT homologies were determined by a BLAST 2 sequences alignment using standard parameters. "ns" refers to a nonsignificant alignment (BLOSUM 62). "nt" refers to enzymatic activities that have not been tested.

small intestine, brain, and kidney of other species. In the species which is most often used as an experimental model resembling man—the mouse—there is clearly no prostatic expression of the murine homolog of PSMA (termed MoPSM), as determined by northern analysis and RT-PCR *(2)*. Furthermore, there is only one gene in the mouse, and the MoPSM gene maps to mouse chromosome 7D1-2, which is syntenic with human 11q14 *(2)*. Considering these facts, it is tempting to suggest that the PSMA-like gene contributes to the extraprostatic expression currently attributed to PSMA, and that expression of PSMA in the prostate may somehow put the prostate at high risk for developing mutations and subsequent carcinogenesis.

8. ENZYMATIC ACTIVITY OF PSMA AND RELATED GENES

Recently, PSMA homologs have been cloned from mouse *(2)*, rat *(5)*, and pig *(22)*, in addition to the cloning of more distantly related paralogs of PSMA in humans *(18,51)*. The cloning and comparison of these homologs and paralogs has shed considerable light on some of the activities of PSMA, and provided some insight into which amino acid sequences appear to be crucial in these activities. PSMA has three known activities: a folate poly γ glutamyl carboxypeptidase (folate hydrolase) *(54)*, a NAALADase *(7)* and a dipeptidyl peptidase IV *(51)*. A comparison of these three activities in the PSMA homologs and paralogs is summarized in Table 1.

The folate hydrolase activity of PSMA describes the sequential removal of γ-linked glutamates from conjugated folates and folate analogs such as methotrexate γ-glutamate

and pteroylpentaglutamate, as shown in Fig. 3A *(54)*. The folate hydrolase activity of PSMA is maintained in the presence of sulfhydryl reducing agents and *p*-hydroxymercuribenzonate, in contrast to an unrelated folate hydrolase enzyme that is located in lysosomes.

PSMA also possesses NAALADase activity, because it is able to hydrolyze the neuropeptide N-acetyl-L-aspartyl-L-glutamate (NAAG) to form *N*-acetyl-L-aspartate and glutamate. This is hydrolysis of the aspartyl α linkage, as shown in Fig. 3B. This activity was first reported by Robinson et al. *(57)* in 1986 in the rat brain, and was demonstrated to be inhibited by quisqualate. NAALADase and its neuropeptide substrate NAAG have been implicated in the regulation of excitatory signaling in the nervous system *(14,77)*. Altered activity of NAALADase has been associated with various neurolgical disorders, including schizophrenia *(11,73)*, Alzheimer's disease, and Huntington's disease *(52)*. In addition, increased levels of NAALADase have been observed in animal models for epilepsy *(45–47,52)* and amylotrophic lateral sclerosis *(58,72,74)*.

Carter et al. *(7)* used antisera to purified rat NAALADase to screen a rat-brain expression library, resulting in the isolation of a partial 1428 nt cDNA clone that had 86% homology to part of the human PSMA cDNA sequence. The entire rat PSMA/NAALADase sequence was subsequently cloned by *(5)*, and when transiently transfected into PC-3 cells (which are NAALADase-negative), they gained NAALADase activity that could be inhibited by quisqualic acid. Human PSMA was also demonstrated to have NAALADase activity *(7)*. Further characterization by Luthi-Carter et al. demonstrated that the human brain NAALADase could be immunoprecipitated with the MAb 7E11-C5 *(44)*. As this antibody binds to residues not conserved in PSMA-like (O'Keefe et al., unpublished), it suggests that human NAALADase and PSMA are derived from the same gene. In addition, Luthi-Carter amplified RNA by RT-PCR, a sequence identical to the LNCaP-derived PSMA sequence, from human cerebellum, indicating that this RNA is present, but in itself not proving that all of the NAALADase activity found in the brain is from PSMA.

Recently, it was shown that PSMA also has dipeptidyl peptidase IV activity, which refers to the ability to hydrolyze Glycine-Proline-7-amido-4-methylcoumarin *(51)*. This amino dipeptidyl peptidase IV activity cleaves the bond between the proline residue and amido methylcoumarin molecule, as shown in Fig. 3C. It was first reported that PSMA possesses this activity when Pangalos et al. *(51)* transiently transfected COS cells with PSMA cDNA and assayed for the dipeptidyl peptidase IV activity. Although the mock-transfected COS cells had dipeptidyl peptidase IV activity, the PSMA-transfected COS cells had significantly more activity. The physiolgical role of the dipeptidyl peptidase IV activity of PSMA is unclear; however, it may play a role in the regulation of various biologically active peptides, including collagen, neuropeptide Y, and growth hormone releasing factor *(51)*. As such, it would appear that PSMA is a multifunctional enzyme, possessing both amino and carboxy-peptidase activities as a mono and dipeptidase.

Rawlings and Barrett predicted the secondary structure of PSMA using a number of protein prediction and protein alignment programs, modifying the results so that the potential zinc ligand binding sites and other blocks of secondary structures were aligned

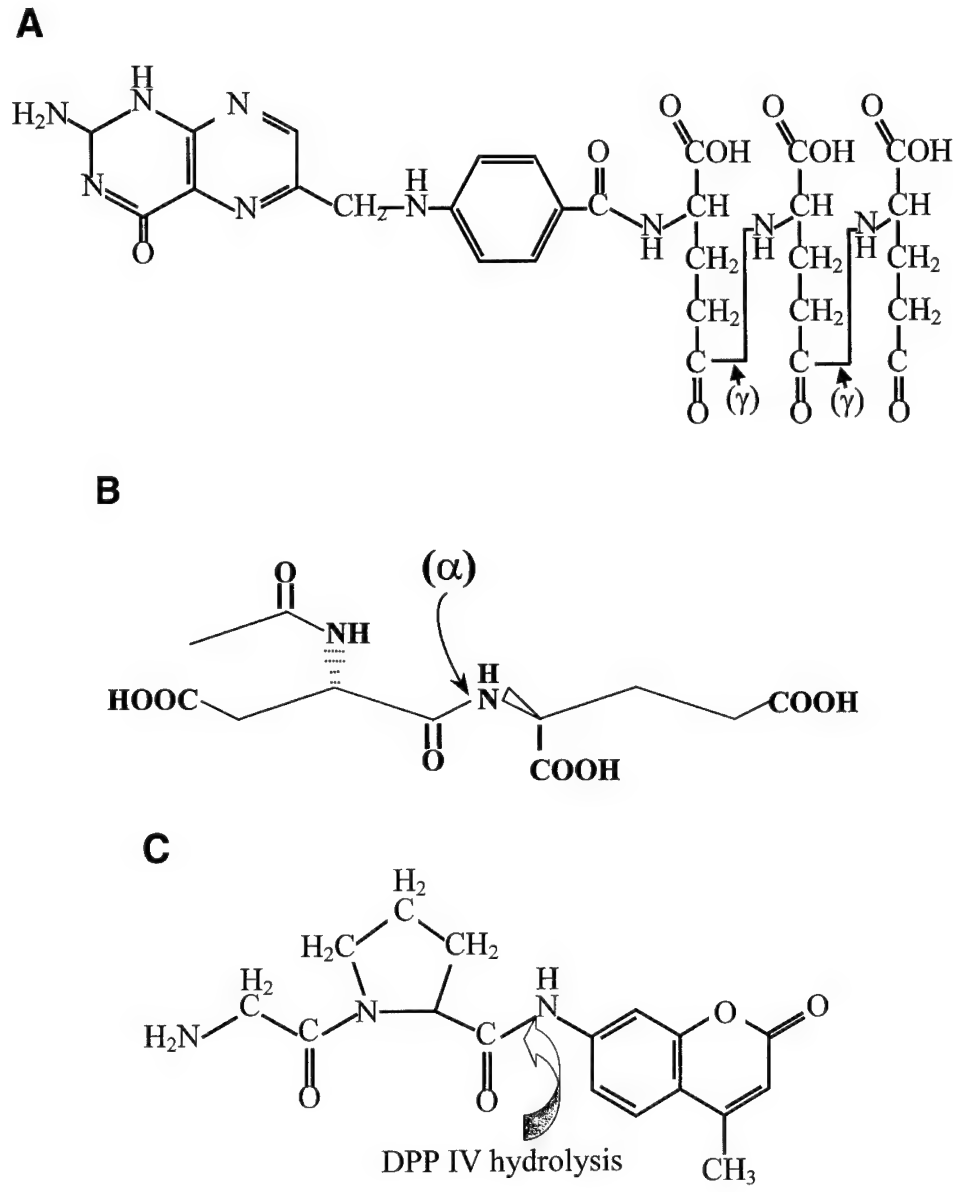

Fig. 3. PSMA substrates. **(A)** folic-acid polyglutamate, **(B)** *N*-acetyl-aspartylglutamate (NAAG), and **(C)** glycine-proline-7-amido-4-methylcoumarin. Arrows indicate the bond that is hydrolyzed by PSMA.

(55). They predicted that PSMA is made up of six organizational domains, with domain E (a.a. 273–587) responsible for the catalytic activity. They were then able to assign this catalytic domain to the M28 peptidase family, and predicted that Asp^{377}, Asp^{387}, Glu^{425}, Asp^{453} and His^{553} are ligands for two atoms of zinc required for catalytic activity *(55)*. Speno et al. performed site-directed mutagenesis experiments alter-

ing these residues resulting in severely reduced NAALADase activity, and were also able to demonstrate that substitution of some amino acids near the putative zinc ligands has a major impact on enzyme structure and/or function *(63)*.

9. THE ROLE OF PSMA IN PROSTATE CARCINOGENESIS AND PROGRESSION

PSMA is believed to be involved both in glutamatergic signaling and folate metabolism. Although there is no evidence that the PSMA substrate NAAG is present in the prostate, our laboratory has demonstrated the existence of Glu 2/3 and Glu 4 glutamate receptors using immunohistochemistry *(26)*. This would suggest that when PSMA is expressed on the surface of the prostate epithelial cell—and particularly when the membrane-bound form of PSMA is upregulated in cancer—sufficient quantities of glutamate could be released to stimulate these receptors, leading to oxidative stress and subsequent cell and DNA damage *(12)*, and further enhancing the ability of the cell to mutate and the cancer to progress.

Another possible function of PSMA was recently reported by Liu et al. *(41)*, who observed a di-leucine motif in the amino terminal of the protein, indicating a possible role in internalization of ligands via PSMA. Incubation of LNCaP cells with antibodies against PSMA followed by laser scanning confocal microscopy revealed that the antibodies were internalized and remained in endosomes within the cell. The endocytosis occurred via clathrin-coated pits and was shown to occur constitutively, although it was enhanced by the presence of antibodies, suggesting a role for PSMA in the internalization of as yet undefined ligand(s) *(41)*.

Our most favored theory is based on the folate hydrolase activity of PSMA and PSM′, which releases the terminal gamma-linked glutamates from folates. To appreciate how the folate hydrolase activity of PSMA might be involved in carcinogenesis and the progression of prostate cancer, it is first necessary to understand the role of folate in this tissue. Dietary folates are generally polygammaglutamated. However, folate can only enter the cell by passive diffusion if it has been deglutamated (although most extracellular folate is monoglutamated). Within the cell, folate is polyglutamated so that it cannot diffuse out of the cell. The presence of PSM′ in the cell would lead to deglutamation of the polyglutamated folate, and subsequent loss of folate from the cell. In the prostate, there is an increased need for folate relative to that of other tissues *(24)*. Folate hydrolase would be expected to deglutamate folate, and allow it to be in a form that could easily diffuse out of the cell—thus placing the cell at risk of becoming folate deficient *(54)*. Folate deficiency is associated with DNA damage and carcinogenesis *(13,34)*.

Folic-acid deficiency can lead to DNA damage via increased uracil incoporation, resulting in single-stranded DNA breaks and decondensation of chromosomes *(4,37)*. Folate deficiency may also lead to carcinogenesis by reducing DNA methylation, which in turn has been proven to lead to the overexpression of certain genes, including a number of oncogenes *(3,16,35)*. Folate is integral to a number of basic metabolic processes in the cell, including DNA synthesis, DNA methylation, and the formation of methionine and polyamines. The prostate is the major organ responsible for polyamines, producing between eightfold and 100-fold greater amounts than other polyamine-producing tissues *(24)*. This high production of polyamines places stress on the folate-

methionine pathway, and as a result the prostate is at greater risk of DNA damage induced by a low-folate environment.

Therefore it is our hypothesis that PSM′, the cytosolic version of PSMA expressed in normal prostate epithelial cells, would be a "catalyst" of DNA damage, and subsequently carcinogenesis, by depleting the prostate of intracellular folate. PSMA, the membrane-bound isoform highly expressed in the tumor and metastatic deposits, could also be expected to hydrolyze poly-γ-glutamated folates, allowing them to diffuse into cells in the local microenvironment. Although poly-γ-glutamated folates are not typically considered extracellular substances, there are a large number of dead or dying cells that can liberate these polyglutamated folates within an environment such as a prostate tumor. Therefore, cells expressing a membrane folate hydrolase such as PSMA would have a growth and survival advantage over nonexpressing cells, especially if the levels of PSM′ decreased in the PSMA-expressing cells. A possible extension of this hypothesis might explain why the neovasculature of most solid tumors express PSMA, tumors which characteristically have an inadequate blood supply may be able to sequester folate from dead cells if they can induce the endothelial cells of the vasculature to express such a folate hydrolase.

After the switch of mRNA splicing to predominantly form PSMA—the membrane-bound isoform of the protein—folate uptake by the cell would be enhanced. This in turn would lead to a greater proliferation rate for the cell, and could possibly also lead to enhanced mutation rates via glutamate receptors and oxidative stress on the cell. In this case, increased expression of PSMA could assist in evolution of the tumor and tumor growth, and progression of the cancer.

To assess this theory in the laboratory, we are currently using the transgenic mouse model. This is significantly assisted by the fact that the murine prostate does not express the homolog of PSMA, MoPSM. We have created transgenic mice expressing human PSMA and/or PSM′ under the control of a prostate-specific promoter, and are examining the effect of folate deficiency on the rate of DNA damage in the presence of the PSMA isoforms (Bacich et al., unpublished).

10. CLINICAL UTILITY OF PROSTATE SPECIFIC MEMBRANE ANTIGEN

The potential of PSMA as a marker of clinical progression was first noted during characterization of the 7E11C5.3 antibody *(28)*. Sera from 20 of 43 patients with prostate cancer appeared to carry molecules reactive with the 7E11C5.3 antibody. However, none of the 30 normal blood donors or seven patients with BPH exhibited such reactivity. The authors also reported that prostate cancer patients who tested positive were more likely to be in progression ($p < 0.05$) *(28)*, although other groups have been unable to detect PSMA in the serum of any patients with metastatic disease *(69)*. The possibility that PSMA could be used as a marker of circulating prostate cancer cells was quickly examined by a number of groups. Israeli et al. *(31)* developed a highly sensitive technique using reverse-transcriptase PCR with nested primer sets to amplify PSMA sequences from patient blood samples. Using similar nested, "enhanced," or radioactive PCR-based methods, the consensus appears to be that there is no correlation between PSMA-positive results and clinical stage, pathological stage, or tumor

grade *(6,49,62)*. There are other reports of PSMA-mRNA expression in normal lymphocytes, urine, and bone marrow (including specimens from female controls), as the result of "illegitimate transcription"—insignificant numbers of PSMA transcripts produced to have any functional effect, but that are able to be detected by sensitive PCR techniques *(10,15,39,80)*.

As with all PCR-based methods to detect circulating cancer cells, the technique needs to be standardized between laboratories. It is clear from the literature that in the hands of different researchers, significant variation is found in test results. Furthermore, the presence of circulating cells does not appear to be directly related to metastatic potential of the primary tumor. For example, Loric et al. *(43)* have shown that patients with inflamed prostates also exhibit circulating prostate cells. However, new technologies currently available may be able to solve these problems. The advent of "Real-Time" PCR, which allows sensitive quantitation of PCR products and the expanding access to custom-designed "Gene Chips" should make it easier to quantitate one or a number of prostate specific transcripts. It has already been shown that a combination of RT-PCR methods for PSMA and PSA-expressing cells is more accurate than either technique alone *(20,79)*. In addition to combining several different markers for analysis, it might also be worthwhile to examine relative amounts of the PSMA mRNA splice variants—particularly PSM′ vs PSMA.

PSMA RT-PCR—in combination with PSA RT-PCR—has also been used to determine the "molecular surgical margins" at radical prostatectomy, by examining five biopsy specimens from the prostatic fossa *(65)*. The results, although preliminary, are promising. The authors found a perfect correlation between a positive PCR result and histopathological determination of positive margins or extracapsular extension. Furthermore, control biopsy specimens taken from men undergoing radical cystoprostatectomy for bladder cancer or abdominoperineal resection for rectal cancer were all negative for the test. Interestingly, in four of 16 cases with histopathologically negative surgical margins, the molecular margins were positive *(65)*. Validation of this unique method requires larger, longer, and multi-institutional studies.

At the present time, clinical imaging using PSMA-directed immunoconjugates utilize the Cyt-356 antibody, commercially known as the "Prostascint Scan™". While the results are promising, the test is probably not optimally designed. In studies localizing the target epitope of Cyt-356, Troyer et al. *(71)* found that the antibody binds to the short cytoplasmic domain of the protein. As such, Cyt-356 binds efficiently to dead cells, and not viable cells *(40,70,71)*. The ability of Cyt-356 to image metastatic deposits is most likely caused by necrotic cells in the tumors, and therefore the sensitivity of imaging would be expected to be enhanced through the use of antibodies directed against the external domain of PSMA. A number of groups have developed such "second generation" antibodies *(40,48)*, and are currently carrying out phase I trials using the antibodies as both imaging agents and therapeutic vectors.

11. THE FUTURE OF CLINICAL AND THERAPEUTIC STRATEGIES UTILIZING PSMA

In an immunotherapeutic approach, Tjoa et al. *(66)* showed that T-cell proliferation could be induced in vitro by autologous dendritic cells pulsed with peptides from the PSMA amino acid sequence. Dendritic cells are professional antigen-presenting cells

that can induce T-cell proliferation and cytotoxicity against specific antigens. This study was followed by Phase I and II clinical trials, which showed positive results, with partial responders identified in groups of patients with both metastatic and suspected local recurrent disease. Follow-up of the responsive patients nearly 300 d later revealed that more than 50% of the subjects were still responding *(59,60,67,68)*. While these results indicate a promising future for immunotherapeutic strategies against prostate cancer, there are several intrinisic problems. The therapy described here is restricted to patients of major histocompatibility antigen type A2 (HLA-A2) tissue type. Furthermore, HLA antigens are downregulated by tumor cells, and thus would not be available for immuno-targeting.

Using an innovative approach to avoid these restrictions, Gong et al. *(19)* devised an immunotherapeutic method that completely circumvents the need for MHC-mediated presentation of peptides. An artificial T-cell receptor was generated by cloning the DNA sequence responsible for recognition of PSMA by the J-591 antibody described here *(40)*, followed by a linker region and the zeta chain receptor, into a retroviral vector *(19)*. T-cells (CD4+ and CD8+) from prostate cancer patients were then transduced with the vector, and their response to cells expressing PSMA was examined. The transduced cells efficiently and specifically lysed PSMA-expressing cells, and also released cytokines in response to PSMA, suggesting that a prolonged response might be feasible *(19)*. If these results are as impressive in vivo as they are in vitro, such an approach should be able to target both the primary tumor and metastatic deposits, as well as the neovasculature of other solid tumors.

Other therapeutic approaches targeting PSMA currently being investigated by our and other laboratories include the use of prodrug strategies and gene therapy. To investigate prodrug strategies against prostate cancer, NIH3T3+/– PSMA and PC-3+/– PSMA-transfected cells and LNCaP cells were grown in the presence of methotrexate triglutamate *(27)* and Heston et al. (unpublished). In the cells expressing PSMA, the drug was converted into its cytotoxic derivative methotrexate, and cell growth was inhibited. However, in the non-PSMA-expressing cells, the drug was nontoxic. While these results show promise, we are currently using the LNCaP xenograft model to determine whether toxicity is specifically targeted to the tumor, or affects other cells expressing the murine homologs of the genes described in Table 1.

Cloning of the PSMA promoter, and more particularly the enhancer, has made the use of gene therapy constructs carrying either therapeutic or cytotoxic genes a viable alternative to those described here *(50a)*. In this approach, cytotoxic genes such as the cytosine deaminase (CD) or herpes virus TK genes are linked to the PSME and a compatible promoter, and the patient is treated with cytotoxic prodrugs such as 5-fluorocytosine or gancyclovir. However, the first challenge is to demonstrate prostate-specificity of the PSME in the transgenic mouse model, and we are currently evaluating this process (Bacich et al., unpublished).

12. INTO THE 21ST CENTURY

The PSMA story has yielded several unexpected surprises so far, but we still do not know whether expression of this gene influences the development or progression of prostate cancer and if so, how. The possible role of PSMA in the angiogenic pathway of tumors is intriguing, but it also suggests that we have much to learn about this fasci-

nating protein. The expression of PSMA in tumor-associated vasculature, as well as its high expression in virtually all prostate tumors and metastases, and particularly in hormone refractory disease for which there is currently no efficient treatment, indicates that targeting of PSMA may be highly valuable as a treatment for not only prostate cancer, but several types of solid tumors.

ACKNOWLEDGMENT

This work was supported in part by a grant from the AFUD/AUA Research Scholar Program and the CR Bard Foundation (D.S. O'Keefe) AFUD/AUA and the Yamanouchi Foundation (D. J. Bacich), as well as grants PC990017 (D.S. O'Keefe), and DAMD17-00-1-0043, DAMD17-99-1-9523 (W.D.W. Heston) from the US Army Medical Research Acquisition Activity, Ft. Detrick, MD; and NIH grants CA29502-16 (D.J. Bacich) and DK/CA47650 (W.D.W. Heston). The information contained within this manuscript does not necessarily reflect the position or policy of the US government.

REFERENCES

1. Antequera, F. and A. Bird. 1995. Number of CpG islands and genes in human and mouse. *Proc. Natl. Acad. Sci. USA* **90:** 11,995–11,999.
2. Bacich, D. J., J. T. Tong, W. P. Heston, and J. T. Pinto. Cloning, expression, genomic localization and glutamyl carboxypeptidase activities of the murine homologue of prostate-specific membrane antigen / NAALADase. *Mammalian Genome* (in press).
3. Bestor, T. H. and B. Tycko. 1996. Creation of genomic methylation patterns. *Nat. Genet.* **12:** 363–367.
4. Blount, B. C. and B. N. Ames. 1994. Analysis of uracil in DNA by gas chromatography-mass spectrometry. *Anal. Biochem.* **219:** 195–200.
5. Bzdega, T., T. Turi, B. Wroblewska, D. She, H. S. Chung, H. Kim, et al. 1997. Molecular cloning of a peptidase against N-acetylaspartylglutamate from a rat hippocampal cDNA library. *J. Neurochem.* **69:** 2270–2277.
6. Cama, C., C. A. Olsson, A. J. Raffo, H. Perlman, R. Buttyan, K. O'Toole, et al. 1995. Molecular staging of prostate cancer. II. A comparison of the application of an enhanced reverse transcriptase polymerase chain reaction assay for prostate specific antigen versus prostate specific membrane antigen. *J. Urol.* **153:** 1373–1378.
7. Carter, R. E., A. R. Feldman, and J. T. Coyle. 1996. Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc. Natl. Acad. Sci. USA* **93:** 749–753.
8. Chang, S. S., D. S. O'Keefe, D. J. Bacich, W. D. W. Heston, V. E. Reuter, and P. B. Gaudin. 1999. prostate-specific membrane antigen (PSMA) is produced in tumor-associated neovasculature. *Clin. Cancer Res.* **5:** 2674–2681.
9. Chang, S. S., V. E. Reuter, W. D. W. Heston, N. H. Bander, L. S. Grauer, and P. B. Gaudin. 1999. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. *Cancer Res.* **59:** 3192–3198.
10. Clements, J. A., P. Rohde, V. Allen, V. J. Hyland, M. L. Samaratunga, W. D. Tilley, et al. 1999. Molecular detection of prostate cells in ejaculate and urethral washings in men with suspected prostate cancer. *J. Urol.* **161:** 1337–1343.
11. Coyle, J. T. 1997. *Neurobiological Disorders* **4:** 231–238.
12. Coyle, J. T. and P. Puttfarcken. 1993. Oxidative stress, glutamate and neurodegenerative disorders. *Science* **262:** 689–695.
13. Eto, I. and C. L. Krumdiek. 1986. Role of vitamin B12 and folate deficiencies in carcinogenesis. *Adv. Exp. Med. Biol.* **206:** 313–330.

14. Fuhrman, S., M. Palkovits, M. Cassidy, and J. H. Neale. 1994. The regional distribution of N-acetylaspartylglutamate (NAAG) and peptidase activity against NAAG in the rat nervous system. *J. Neurochem.* **62:** 275–281.
15. Gala, J. L., M. Heusterspreute, S. Loric, F. Hanon, B. Tombal, P. Van Cangh, et al. 1998. Expression of prostate-specific antigen and prostate-specific membrane antigen transcripts in blood cells: implications for the detection of hematogenous prostate cells and standardization. *Clin. Chem.* **44:** 472–481.
16. Ghoshal, A. K. and E. Farber. 1984. The induction of liver cancer by dietary dificiency of choline and methionine without added carcinogens. *Carcinogenesis* **5:** 1367–1370.
17. Giebel, L. B., K. M. Strunk, and R. A. Spritz. 1991. Organization and nucleotide sequences of the human tyrosinase gene and a truncated tyrosinase-related segment. *Genomics* **9:** 435–445.
18. Gingras, R., C. Richard, M. El-Alfy, C. R. Morales, M. Potier, and A. V. Pshezhetsky. 1999. Purification, cDNA cloning, and expression of a new human blood plasma glutamate carboxypeptidase homologous to N-acetyl-aspartyl-alpha-glutamate carboxypeptidase/prostate-specific membrane antigen. *J. Biol. Chem.* **274:** 11,742–11,750.
19. Gong, M. C., J. B. Latouche, A. Krause, W. D. W. Heston, N. H. Bander, and M. Sadelain. 1999. Cancer patient T cells genetically targeted to prostate-specific membrane antigen specifically lyse prostate cancer cells and release cytokines in response to prostate-specific membrane antigen. *Neoplasia* **1:** 123–127.
20. Grasso, Y. Z., M. K. Gupta, H. S. Levin, C. D. Zippe, and E. A. Klein. 1998. Combined nested RT-PCR assay for prostate-specific antigen and prostate-specific membrane antigen in prostate cancer patients: correlation with pathological stage. *Cancer Res.* **58:** 1456–1459.
21. Grauer, L. S., K. D. Lawler, J. L. Marignac, A. Kumar, A. S. Goel, and R. L. Wolfert. 1998. Identification, purification, and subcellular localization of prostate-specific membrane antigen PSM′ protein in the LNCaP prostatic carcinoma cell line. *Cancer Res.* **58:** 4787–4789.
22. Halsted, C. H., E. H. Ling, R. Luthi-Carter, J. A. Villanueva, J. M. Gardner, and J. T. Coyle. 1998. Folylpoly-gamma-glutamate carboxypeptidase from pig jejunum. Molecular characterization and relation to glutamate carboxypeptidase II. *J. Biol. Chem.* **273:** 20,417–20,424.
23. Hasenson, M., B. Lundh, R. Stege, K. Carlstrom, and A. Pousette. 1989. PAP and PSA in prostatic carcinoma cell lines and aspiration biopsies: relation to hormone sensitivity and to cytological grading. *Prostate* **14:** 83–90.
24. Heston, W. D. W. 1991. Prostatic polyamines and polyamine targeting as a new approach to therapy of prostate cancer, in *Prostate Cancer Cell and Molecular Mechanisms in Diagnosis and Treatment* (Isaacs, J. T., ed.), Cold Spring Harbor Laboratory Press, Cold spring Harbor, NY.
25. Heston, W. D. W. 1997. Biologic implications for prostatic function following identification of prostate-specific membrane antigen as a novel folate hydrolase/neurocarboxypeptidase, in *Prostate: Basic and Clinical Aspects* (Naz, R. K., ed.), CRC Press: pp. 267–298.
26. Heston, W. D. W., D. A. Silver, I. Pellicer, W. R. Fair, C. Cordon-Cardo. 1996. Ionotropic glutamate receptor distribution in prostate tissue. *J. Urol.* **155:** 513A.
27. Heston, W. D. W., W-P. Tong, and J. T. Pinto. 1997. Prostate-specific membrane antigen: a unique folate hydrolase: potential target for prodrug therapy. *Mol. Urol.* **1:** 215–219.
28. Horoszewicz, J. S., E. Kawinski, and G. P. Murphy. 1987. Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Res.* **7:** 927–935.
29. Horoszewicz, J. S., S. S. Leong, T. M. Chu, Z. L. Wajsman, M. Friedman, L. Papsidero, et al. 1980. The LNCaP cell line—a new model for studies on human prostatic carcinoma. *Prog. Clin. Biol. Res.* **37:** 115–132.
30. Horoszewicz, J. S., S. S. Leong, E. Kawinski, J. P. Karr, H. Rosenthal, T. M. Chu, et al. 1983. LNCaP model of human prostatic carcinoma. *Cancer Res.* **43:** 1809–1818.

31. Israeli, R. S., W. H. Miller, Jr., S. L. Su, C. T. Powell, W. R. Fair, D. S. Samadi, et al. 1994. Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostatic tumor cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. *Cancer Res.* **54:** 6306–6310.
32. Israeli, R. S., C. T. Powell, J. G. Corr, W. R. Fair, and W. D. W. Heston. 1994. Expression of the prostate-specific membrane antigen. *Cancer Res.* **54:** 1807–1811.
33. Israeli, R. S., C. T. Powell, W. R. Fair, and W. D. Heston. 1993. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. *Cancer Res.* **53:** 227–230.
34. Jennings, E. 1995. Folic acid as a cancer-preventing agent. *Med. Hypotheses* **45:** 297–303.
35. Jones, P. A. and J. D. Buckley. 1990. The role of methylation in cancer. *Adv. Cancer Res.* **54:** 1–23.
36. Kawakami, M. and J. Nakayama. 1997. Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by *in situ* hybridization. *Cancer Res.* **57:** 2321–2324.
37. Krumdiek, C. L. and P. N. Howard-Peebles. 1983. On the nature of folic acid-sensitive fragile sites in human chromosomes: an hypothesis. *Am. J. Med. Genet.* **16:** 23–28.
38. Leek, J., N. Lench, B. Maraj, A. Bailey, I. M. Carr, S. Andersen, et al. 1995. Prostate-specific membrane antigen: evidence for the existence of a second related human gene. *Br. J. Cancer* **72:** 583–588.
39. Lintula, S. and U. H. Stenman. 1997. The expression of prostate-specific membrane antigen in peripheral blood leukocytes. *J. Urol.* **157:** 1969–1972.
40. Liu, H., P. Moy, S. Kim, Y. Xia, A. Rajasekaran, V. Navarro, et al. 1997. Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. *Cancer Res.* **57:** 3629–3634.
41. Liu, H., A. K. Rajasekaran, P. Moy, Y. Xia, S. Kim, V. Navarro, et al. 1998. Constitutive and antibody-induced internalization of prostate-specific membrane antigen. *Cancer Res.* **58:** 4055–4060.
42. Lopes, A. D., W. L. Davis, M. J. Rosenstraus, A. J. Uveges, and S. C. Gilman. 1990. Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from antiprostate monoclonal antibody 7E11-C5. *Cancer Res.* **50:** 6423–6429.
43. Loric, S., F. Dumas, P. Eschwege, P. Blanchet, G. Benoit, A. Jardin, et al. 1995. Enhanced detection of hematogenous circulating prostatic cells in patients with prostate adenocarcinoma by using nested reverse transcription polymerase chain reaction assay based on prostate-specific membrane antigen. *Clin. Chem.* **41:** 1698–1704.
44. Luthi-Carter, R., A. K. Barczak, H. Speno, and J. T. Coyle. 1998. Molecular characterization of human brain N-acetylated alpha-linked acidic dipeptidase (NAALADase). *J. Pharmacol. Exp. Ther.* **286:** 1020–1025.
45. Meyerhoff, J. L., R. E. Carter, D. L. Yourick, B. S. Slusher, and J. T. Coyle. 1992. Activity of a NAAG-hydrolyzing enzyme in brain may affect seizure susceptibility in genetically epilepsy-prone rats. *Epilepsy Res. Suppl.* **9:** 163–172.
46. Meyerhoff, J. L., R. E. Carter, D. L. Yourick, B. S. Slusher, and J. T. Coyle. 1992. Genetically epilepsy-prone rats have increased brain regional activity of an enzyme which liberates glutamate from N-acetyl-aspartyl-glutamate. *Brain Res.* **593:** 140–143.
47. Meyerhoff, J. L., M. B. Robinson, M. A. Bixler, S. S. Richards, and J. T. Coyle. 1989. Seizures decrease regional enzymatic hydrolysis of N-acetyl-aspartylglutamate in rat brain. *Brain Res.* **505:** 130–134.
48. Murphy, G. P., T. G. Greene, W. T. Tino, A. L. Boynton, and E. H. Holmes. 1998. Isolation and characterization of monoclonal antibodies specific for the extracellular domain of prostate specific membrane antigen. *J. Urol.* **160:** 2396–2401.

49. Noguchi, M., J. Miyajima, K. Itoh, and S. Noda. 1997. Detection of circulating tumor cells in patients with prostate cancer using prostate specific membrane-derived primers in the polymerase chain reaction. *Int. J. Urol.* **4:** 374–379.
50. O'Keefe, D. S., S. L. Su, D. J. Bacich, Y. Horiguchi, Y. Luo, C. T. Powell, et al. 1998. Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochim. Biophys. Acta* **1443:** 113–127.

50a. O'Keefe, D. S., A. Uchida, D. J. Bacich, F. B. Watt, A. Matorana et al. 2000. Prostate-specific suicide gene therapy using the prostate specific membrane antigen promoter and enhancer. *Prostate* (in press).

51. Pangalos, M. N., J. M. Neefs, M. Somers, P. Verhasselt, M. Bekkers, L. van der Helm, et al. 1999. Isolation and expression of novel human glutamate carboxypeptidases with N-acetylated alpha-linked acidic dipeptidase and dipeptidyl peptidase IV activity. *J. Biol. Chem.* **274:** 8470–8483.
52. Passani, L. A., J. P. Vonsattel, R. E. Carter, and J. T. Coyle. 1997. N-acetylaspartylglutamate, N-acetylaspartate, and N-acetylated alpha-linked acidic dipeptidase in human brain and their alterations in Huntington and Alzheimer's diseases. *Mol. Chem. Neuropathol.* **31:** 97–118.
53. Peace, D. J. Z., Y. Holt, G. Ferrer, et al. 1999. Identification of three novel splice variants of prostate-specific membrane antigen in prostate cancer cells. American Association for Cancer Research 90th Annual Meeting, April 10–14, 1999, Philadelphia, PA.
54. Pinto, J. T., B. P. Suffoletto, T. M. Berzin, C. H. Qiao, S. Lin, W. P. Tong, et al. 1996. Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin. Cancer Res.* **2:** 1445–1451.
55. Rawlings, N. D. and A. J. Barrett. 1997. Structure of membrane glutamate carboxypeptidase. *Biochim. Biophys. Acta* **1339:** 247–252.
56. Rinker-Schaeffer, C. W., A. L. Hawkins, S. L. Su, R. S. Israeli, C. A. Griffin, J. T. Isaacs, et al. 1995. Localization and physical mapping of the prostate-specific membrane antigen (PSM) gene to human chromosome 11. *Genomics* **30:** 105–108.
57. Robinson, M. B., R. D. Blakely, R. Couto, and J. T. Coyle. 1987. *J. Biol. Chem.* **262:** 14,498–14,506.
58. Rothstein, J. D., G. Tsai, R. W. Kuncl, L. Clawson, D. R. Cornblath, D. B. Drachman, et al. 1990. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. *Ann. Neurol.* **28:** 18–25.
59. Salgaller, M. L., P. A. Lodge, J. G. McLean, B. A. Tjoa, D. J. Loftus, H. Ragde, et al. 1998. Report of immune monitoring of prostate cancer patients undergoing T-cell therapy using dendritic cells pulsed with HLA-A2-specific peptides from prostate-specific membrane antigen (PSMA). *Prostate* **35:** 144–151.
60. Salgaller, M. L., B. A. Tjoa, P. A. Lodge, H. Ragde, G. Kenny, A. Boynton, et al. 1998. Dendritic cell-based immunotherapy of prostate cancer. *Crit. Rev. Immunol.* **18:** 109–119.
61. Silver, D. A., I. Pellicer, W. R. Fair, W. D. W. Heston, and C. Cordon-Cardo. 1997. Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin. Cancer Res.* **3:** 81–85.
62. Sokoloff, M. H., C. L. Tso, R. Kaboo, S. Nelson, J. Ko, F. Dorey, et al. 1996. Quantitative polymerase chain reaction does not improve preoperative prostate cancer staging: a clinicopathological molecular analysis of 121 patients. *J. Urol.* **156:** 1560–1566.
63. Speno, H. S., R. Luthi-Carter, W. L. Macias, S. L. Valentine, A. R. Joshi, and J. T. Coyle. 1999. Site-directed mutagenesis of predicted active site residues in glutamate carboxypeptidase II. *Mol. Pharmacol.* **55:** 179–185.
64. Su, S. L., I. P. Huang, W. R. Fair, C. T. Powell, and W. D. W. Heston. 1995. Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression. *Cancer Res.* **55:** 1441–1443.

65. Theodorescu, D., H. F. Frierson, Jr., and R. A. Sikes. 1999. Molecular determination of surgical margins using fossa biopsies at radical prostatectomy. *J. Urol.* **161:** 1442–1448.
66. Tjoa, B., A. Boynton, G. Kenny, H. Ragde, S. L. Misrock, and G. Murphy. 1996. Presentation of prostate tumor antigens by dendritic cells stimulates T-cell proliferation and cytotoxicity. *Prostate* **28:** 65–69.
67. Tjoa, B. A., S. J. Erickson, V. A. Bowes, H. Ragde, G. M. Kenny, O. E. Cobb, et al. 1997. Follow-up evaluation of prostate cancer patients infused with autologous dendritic cells pulsed with PSMA peptides. *Prostate* **32:** 272–278.
68. Tjoa, B. A., S. J. Simmons, A. Elgamal, M. Rogers, H. Ragde, G. M. Kenny, et al. 1999. Follow-up evaluation of a phase II prostate cancer vaccine trial. *Prostate* **40:** 125–129.
69. Troyer, J. K., M. L. Beckett, and G. L. Wright, Jr. 1995. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int. J. Cancer* **62:** 552–558.
70. Troyer, J. K., M. L. Beckett, and G. L. Wright, Jr. 1997. Location of prostate-specific membrane antigen in the LNCaP prostate carcinoma cell line. *Prostate* **30:** 232–242.
71. Troyer, J. K., Q. Feng, M. L. Beckett and G. L. Wright Jr. 1995. Biochemical characterization and mapping of the 7E11C5.3 epitope of the prostate-specific membrane antigen. *Urologic Oncology* **1:** 29–37.
72. Tsai, G., L. C. Cork, B. S. Slusher, D. Price, and J. T. Coyle. 1993. Abnormal acidic amino acids and N-acetylaspartylglutamate in hereditary canine motoneuron disease. *Brain Res.* **629:** 305–309.
73. Tsai, G., L. A. Passani, B. S. Slusher, R. Carter, L. Baer, J. E. Kleinman, et al. 1995. Abnormal excitatory neurotransmitter metabolism in schizophrenic brains. *Arch. Gen. Psychiatry* **52:** 829–836.
74. Tsai, G. C., B. Stauch-Slusher, L. Sim, J. C. Hedreen, J. D. Rothstein, R. Kuncl, et al. 1991. Reductions in acidic amino acids and N-acetylaspartylglutamate in amyotrophic lateral sclerosis CNS. *Brain Res.* **556:** 151–156.
75. Watt, F. B., D. E. Ho, T. Martorana, A. Kingsley, E. O'Keefe, D. S. Russell, et al. 2000. A Tissue-specific enhancer of the prostate-specific membrane antigen gene. *Genomics* (in press).
76. Wright, G. L., Jr., B. M. Grob, C. Haley, K. Grossman, K. Newhall, D. Petrylak, et al. 1996. Upregulation of prostate-specific membrane antigen after androgen- deprivation therapy. *Urology* **48:** 326–334.
77. Wroblewska, B., M. R. Santi, and J. H. Neale. 1998. N-acetylaspartylglutamate activates cyclic AMP-coupled metabotropic glutamate receptors in cerebellar astrocytes. *Glia* **24:** 172–179.
78. Wynant, G. E., G. P. Murphy, J. S. Horoszewicz, C. E. Neal, B. D. Collier, E. Mitchell, et al. 1991. Immunoscintigraphy of prostatic cancer: preliminary results with 111In-labeled monoclonal antibody 7E11-C5.3 (CYT-356). *Prostate* **18:** 229–241.
79. Zhang, Y., C. D. Zippe, F. Van Lente, E. A. Klein, and M. K. Gupta. 1997. Combined nested reverse transcription-PCR assay for prostate-specific antigen and prostate-specific membrane antigen in detecting circulating prostatic cells. *Clin. Cancer Res.* **3:** 1215–1220.
80. Zippelius, A., P. Kufer, G. Honold, M. W. Kollermann, R. Oberneder, G. Schlimok, et al. 1997. Limitations of reverse-transcriptase polymerase chain reaction analyses for detection of micrometastatic epithelial cancer cells in bone marrow. *J. Clin. Oncol.* **15:** 2701–2708.

The Prostate 45:149–157 (2000)

Prostate-Specific Suicide Gene Therapy Using the Prostate-Specific Membrane Antigen Promoter and Enhancer

Denise S. O'Keefe,[1] Atsushi Uchida,[1] Dean J. Bacich,[1] Fujiko B. Watt,[2] Anna Martorana,[2] Peter L. Molloy,[2] and Warren D.W. Heston[1,3]*

[1]*George M. O'Brien Urology Research Center, Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio*
[2]*CSIRO Molecular Science, North Ryde, New South Wales, Australia*
[3]*Department of Urology, Cleveland Clinic Foundation, Cleveland, Ohio*

BACKGROUND. Prostate-specific membrane antigen (PSMA) is abundantly expressed in virtually 100% of prostate cancers and metastases. In addition, unlike prostate-specific antigen (PSA), PSMA is upregulated under conditions of androgen deprivation. Therefore, PSMA is an attractive therapeutic target for advanced prostate cancer. Recently, both the promoter and the enhancer driving prostate-specific expression of the PSMA gene were cloned. We describe here our analysis of the PSMA enhancer for the most active region(s) and present a way of using the enhancer in combination with the *E. coli* cytosine deaminase gene for suicide-driven gene therapy that converts the nontoxic prodrug 5-fluorocytosine (5-FC) into the cytotoxic drug 5-fluorouracil (5-FU) in prostate cancer cells.

METHODS. Deletion constructs of the full-length PSMA enhancer were subcloned into a luciferase reporter vector containing either the PSMA or SV-40 promoter. The most active portion of the enhancer was then determined via luciferase activity in the C4-2 cell line. We then replaced the luciferase gene with the *E. coli* cytosine deaminase gene in the subclone that showed the most luciferase activity. The specificity of this technique was examined in vitro, using the prostate cancer cell line LNCaP, its androgen-independent derivative C4-2, and a number of nonprostatic cell lines. The toxicity of 5-FC and 5-FU on transiently transfected cell lines was then compared.

RESULTS. The enhancer region originally isolated from the PSMA gene was approximately 2 kb. Deletion constructs revealed that at least two distinct regions seem to contribute to expression of the gene in prostate cancer cells, and therefore the best construct for prostate-specific expression was determined to be 1,648 bp long. The IC_{50} of 5-FC was similar in all cell lines tested (>10 mM). However, transfection with the 1648 nt PSMA enhancer and the PSMA promoter to drive the cytosine deaminase gene enhanced toxicity in a dose-dependent manner more than 50-fold, while cells that did not express the PSMA gene were not significantly sensitized by transfection.

CONCLUSIONS. Suicide gene therapy using the PSMA enhancer may be of benefit to pa-

Grant sponsor: U.S. Army Medical Research Acquisition Activity, Ft. Detrick, MD; Grant numbers: DAMD 17-00-1-0043, DAMD17-99-1-9523; Grant sponsor: CaPCURE.

The information contained within this manuscript does not necessarily reflect the position or policy of the U.S. Government.

*Correspondence to: W.D.W. Heston, George M. O'Brien Urology Research Center, Box NB-40, Department of Cancer Biology, Cleveland Clinic Foundation, Cleveland, OH 44195.
E-mail: hestonw@ccf.org

Received 13 June 2000; Accepted 15 June 2000

DSO'K is a C.R. Bard fellow and is supported by an AFUD/AYA fellowship.

tients who have undergone androgen ablation therapy and are suffering a relapse of disease.
Prostate 45:149–157, 2000.

KEY WORDS: **enhancer elements; promoter regions; gene therapy; prodrugs; flucytosine; PSMA**

INTRODUCTION

Prostate cancer is the second leading cause of cancer death [1]. Although the most recent data suggest that the incidence of death from this disease is on the decline due to the advent of the prostate-specific antigen (PSA) test and subsequent early intervention, once a patient becomes hormone-refractory, there are few treatment choices available [1]. One approach currently being considered is prostate-specific gene therapy followed by prodrug administration. The prodrug 5-fluorocytosine (5-FC) is converted by the bacterial enzyme cytosine deaminase (CD) to 5-fluorouracil (5-FU) [2,3]. 5-FU has already been used for treatment of metastatic prostate cancer, while 5-FC is used in the treatment of fungal infections and is nontoxic to humans. Prostate-specific membrane antigen (PSMA) is highly expressed in prostate cancer and normal prostate [4–6], and more recently, expression of PSMA was observed in the tumor-associated neovasculature of nearly all solid tumors [7–10].

Expression of PSMA or a "PSMA-Like" gene has also been seen in some other tissues including kidney, liver, and brain, although at a much lower level than in the prostate or prostatic carcinoma, as determined by Northern blot analyses [6] (also Bacich et al., unpublished observations). Furthermore, expression of PSMA is upregulated under conditions of androgen deprivation, which makes it a useful marker for patients who have undergone hormonal ablation [6]. Accordingly, PSMA is currently being used as a therapeutic and clinical target in a number of strategies against prostate cancer. Recently, our group cloned the PSMA promoter and an enhancer from intron 3 of the PSMA gene that drives prostate-specific expression of reporter genes [11–13]. To further our aim of generating a way of targeting prostate cancer and cancer metastases using gene therapy, we designed a genetic construct that places expression of the bacterial CD gene under the regulatory control of the PSMA promoter and enhancer.

MATERIALS AND METHODS

Cell Culture

The prostate cancer cell line LNCaP, the breast cancer cell line MCF-7, colorectal cancer line HCT8, and lung cancer cell line H157 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The androgen-independent subline of LNCaP, C4-2, was purchased from UroCor Laboratories (Oklahoma City, OK). LNCaP and HCT8 cells were grown in RPMI-1640 (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (FBS). C4-2 and MCF-7 were grown in DMEM (Life Technologies, Rockville, MD) containing 10% FBS. H157 cells were grown in RPMI-1640 with 2 mM L-glutamine, adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate with 10% FBS. All cells were grown in the absence of antibiotics.

Cloning of Enhancer Deletion Constructs

The promoter of the PSMA gene was cloned into the pGL3-Basic vector (Promega, Madison, WI) as previously described, to form pGL3-B-PSM [11]. Deletion constructs of the PSMA enhancer were produced using primers with an artifically incorporated *Bam*HI restriction endonuclease recognition site. The following primer sets were used to amplify genomic DNA NA1944 (Coriell Cell Repositories, Camden, NJ). This DNA was derived from a somatic cell hybrid containing retaining human 11pter→cen translocated to a hamster chromosome, and therefore does not contain the PSMA-like gene. PCR was carried out under standard conditions. Following PCR, the resultant products were digested with *Bam*HI and subjected to gel electrophoresis followed by gel purification. The gel-purified products were cloned into pGL3-B-PSM or pGL3-promoter vector (containing the SV-40 promoter and no enhancer; Promega, Madison, WI), which had been digested with *Bam*HI and treated with alkaline phosphatase. The primer sets used were: S 14704 5′ CGCGGATCCGCCTTCTAAAATGAGTTGGG 3′, with each of the following primers: AS 15205 5′ CGCGGATCCCAACATAGTGGAACCACGTC 3′ (501 bp), AS 15573 5′ CGCGGATCCTGAGAAAAGATTGCCAACGC 3′ (869 bp), AS 15994 5′ CGCGGATCCATTAGGTTCCAAAGGAAGCC 3′ (1,290 bp), AS 16352 5′ CGCGGATCCGGCTACTACATAAGTATAAGTC 3′ (1,648 bp), and AS 16617 5′ CGCGGATCCATGACACCAAAGCTTTAGGG 3′ (1,913 bp). The artifically incorporated *Bam*HI restriction sites are underlined.

Analysis of PSMA Enhancer Constructs

Activity of the PSMA enhancer constructs was determined using luciferase as a reporter gene following transient transfection. C4-2 and MCF-7 cells were used as positive and negative control cells, respectively, to determine activity since tissue specificity of the entire enhancer region has already been demonstrated [13]. The day before transfection, 50,000 C4-2 and 75,000 MCF-7 cells were plated into 24-well plates. Cells were then transfected with 400 ng of each enhancer construct, using Lipofectamine Plus Reagent (Life Technologies), according to the manufacturer's instructions, with the exception that the DNA/lipofectamine complexes were allowed to remain on the cells overnight. Approximately 40 hr after transfection, cells were harvested and analyzed for reporter activity, using the Dual Luciferase Assay Kit (Promega). All experiments were carried out in triplicate, and each well was cotransfected with 100 ng of pBIND (Promega), which is a plasmid carrying the *Renilla* luciferase gene under the control of the SV-40 promoter and enhancer, to control for transfection efficiency.

Cloning of the Cytosine Deaminase Gene Construct

The plasmid pCD containing the cytosine deaminase gene from *E. coli* was kindly provided by Dr. I. Yoshimura (Department of Urology, National Defense Medical College, Saitama, Japan). The gene was modified to possess a eukaryotic translation initiation signal. The luciferase gene in the enhancer construct showing the most reporter gene activity, pGL3-B-PSM-Enh1648, was removed by restriction enzyme digestion with *Xba*I and replaced with the CD gene from pCD (the 1.5-kb CD gene from pCD was excised using *Xba*I and *Spe*I digestion). *Xba*I digestion actually removes some of the PSMA promoter sequence, but the removal of this region has no effect on promoter activity (O'Keefe et al., unpublished observations). Subsequent clones were sequenced to determine the orientation of the CD gene with respect to the PSMA promoter. The clones used for the following experiments were named pPSM/CD-sense and pPSM/CD-antisense, where sense and antisense refer only to the direction of the CD gene.

Cytotoxicity/Proliferation Inhibition Assay

Initially, the cytotoxicity of 5-FC and 5-FU in nontransfected cell lines was examined. Cells (2.5×10^3 C4-2, H157, and HCT8, and 4.0×10^4 LNCaP and MCF-7 cells) were plated in triplicate for each time point, in 24-well plates. The cells were then exposed 24 hr later to various concentrations of 5-FC (Sigma, St. Louis, MO) or 5-FU (Sigma). Relative cell numbers were assayed after 3 days, using the MTS assay (Cell Titer 96 AQ_{ueous}, Promega), and the inhibitory concentration (IC_{50}) was determined by figuring the concentration at which the cell number was 50% of the number of control (untreated) cells grown for the same length of time.

Cytotoxicity of 5-FC on Transiently Transfected Cells

The cytoxicity of 5-FC on cells transiently transfected with pPSM/CD-sense or pPSM/CD-antisense or liposome only was determined. Cells were plated in 24-well plates and transfected as described above. Transfection efficiency was determined by in situ X-gal staining replica wells transfected with pSV-β-galactosidase (Promega). Two days after initiation of transfection, cells were exposed to various concentrations of 5-FC for 3 days, and cell viability was measured.

Enzymatic Activity of Cytosine Deaminase in the Transfected Cells

The enzymatic activity in the cell lysate was measured with spectrophotometry [14]. H157, LNCaP, and C4-2 cells were plated in a 100-mm dish and preincubated for 24–48 hr. At 50% confluency, the cells were transfected with pPSM/CD-sense, pPSM/CD-antisense, or pSV-β-galactosidase. Two days after initiation of transfection, the cells were collected and preserved at −20°C. On the initial day of the assay, 110 μl of PBS were added to each cell pellet. Pellets were then sonicated and centrifuged at 14,000*g* at 4°C. Ten microliters of each supernatant were used to measure the protein concentration, using the BCA protein assay (Pierce, Rockford, IL). The remaining 100 μl of cell lysate were added to 900 μl of 3 mM 5-FC in PBS. The reaction mixtures were incubated at 37°C. At various time points, 50 μl of each aliquot were removed and added to 950 μl of 0.1M HCl. The concentration of 5-FC was measured by determining the absorbance at 290 nm (A290) and 255 nm (A255). The concentration of 5-FC was calculated as follows:

$$\text{5-FC (mM)} = 0.119 \times \text{A290} - 0.025 \times \text{A255}$$ [14].

The enzymatic unit of cytosine deaminase was defined as nmol of 5-FC catalyzed per min per mg of protein from whole-cell supernatant.

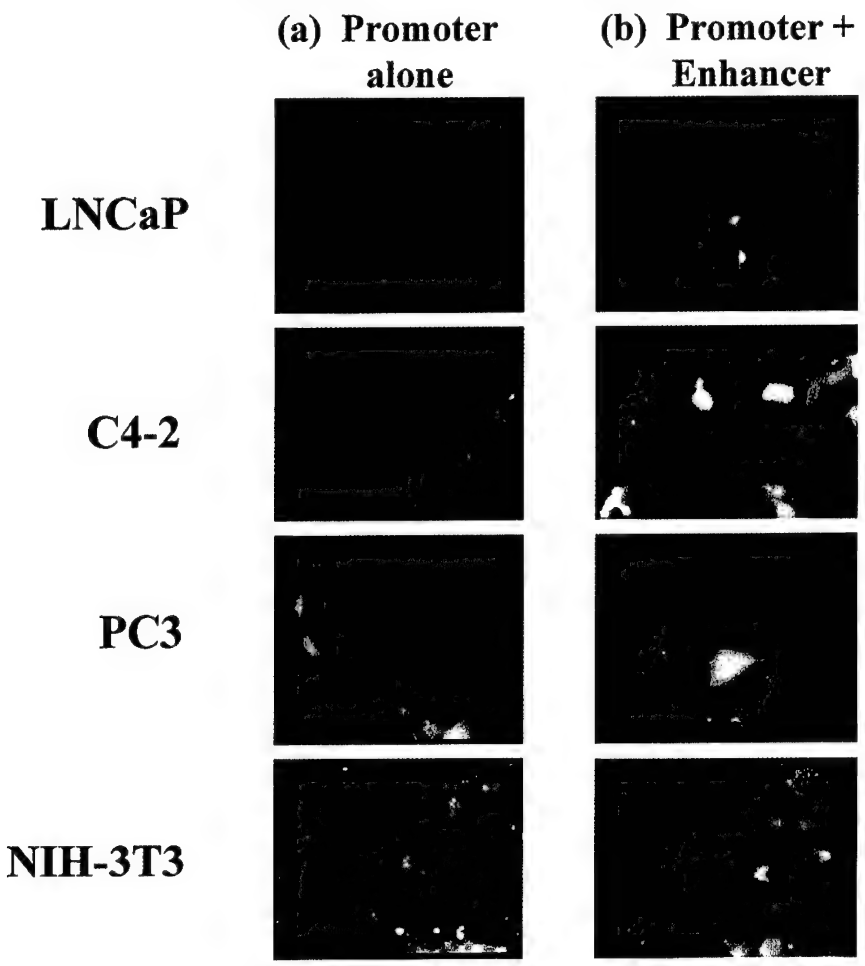

Fig. 1. PMSA promoter and promoter/enhancer-driven expression of GFP **a:** PSMA promoter alone. **b:** PSMA promoter and enhancer, driving GFP expression in LNCaP, C4-2, and PC-3 (prostatic cell lines) and in NIH 3T3 cells (used as a negative control). Addition of the PSMA enhancer to the PSMA promoter clearly results in a large increase in GFP reporter expression in LNCaP (magnification ×200, 30-sec exposure) and C4-2 (magnification ×400, 30-sec exposure) cells, while no expression is seen in NIH-3T3 (magnification ×400, 60-sec exposure) cells. Note the fluorescence of the promoter alone in PC-3 (magnification ×400, 30-sec exposure) cells, which do not express PSMA.

RESULTS

PSMA Promoter and Promoter/Enhancer-Driven GFP Expression in Various Cell Lines

Green fluorescent protein (GFP) expression was used to compare the activity of the PSMA promoter with that of the PSMA promoter and the PSMA enhancer that was identified by Watt et al. [13]. These constructs were transiently transfected into LNCaP, C4-2, PC-3, and NIH-3T3 cell lines, and the cells were examined for green fluorescence (see Fig. 1). Minimal GFP expression was seen in LNCaP, C4-2, and NIH-3T3 cells with the promoter alone, though PC-3 cells had moderately high levels of GFP expression with the PSMA promoter alone. Addition of the PSMA enhancer to the construct resulted in significantly increased levels of GFP expression in the LNCaP and C4-2 cell lines, although there appeared to be no enhancement of GFP expression in the PC-3 and NIH-3T3 cells over that seen with the PSMA promoter alone.

Analysis of the PSMA Enhancer

Reporter plasmids carrying various portions of the PSMA enhancer and driven by either the PSMA promoter or the viral SV-40 promoter were analyzed for luciferase expression in the prostatic cell line C4-2 and the breast cancer cell line MCF-7. The entire enhancer sequence is shown in Figure 2A, as well as the constructs indicated by the primer positioning, so that all deletions were made progressively from the 3′ end of the enhancer (see Fig. 2B). All the enhancer deletion constructs were tested with the enhancer placed in both orientations (downstream of the luciferase reporter gene) with respect to the PSMA or SV-40 pro-

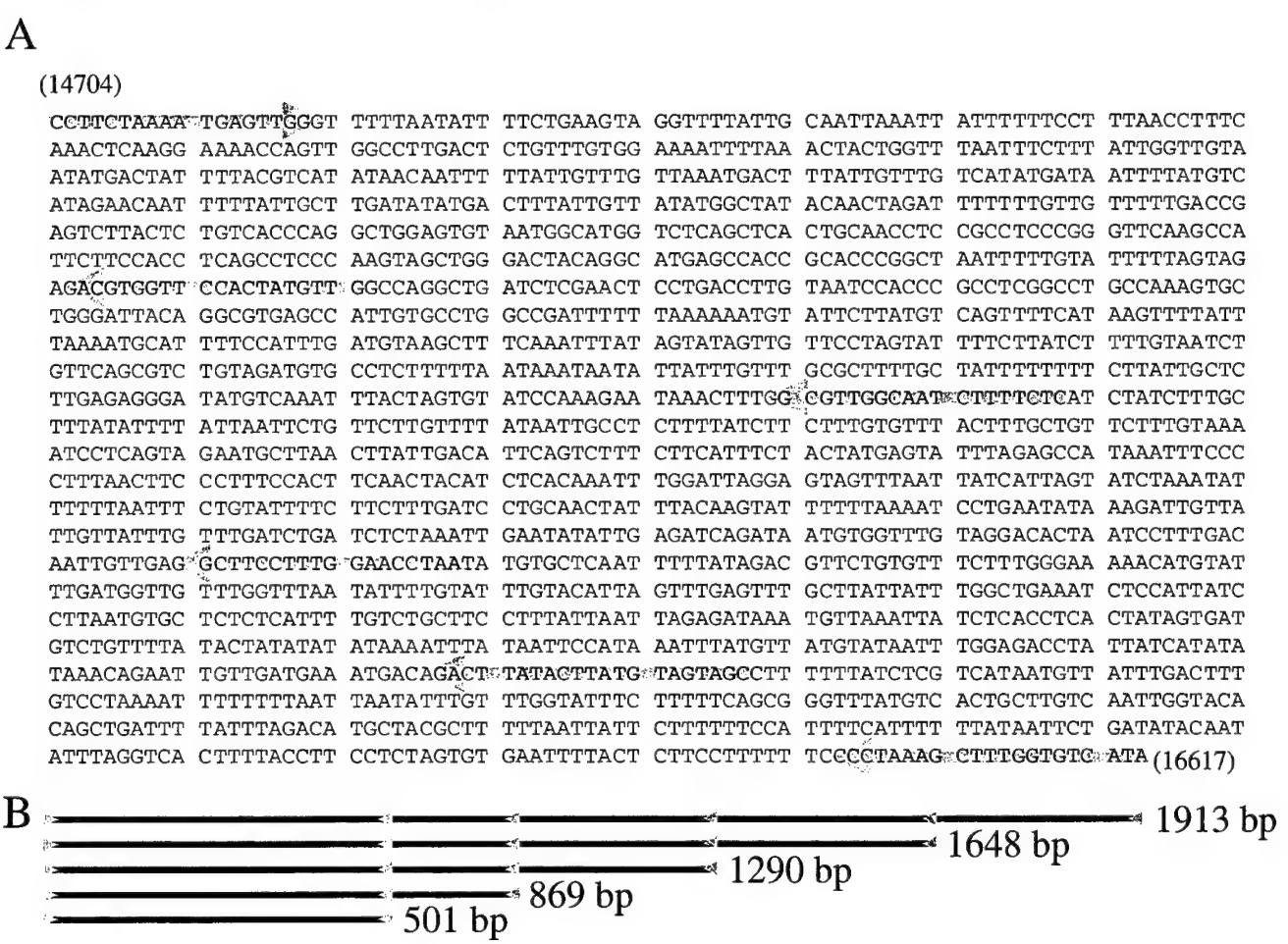

Fig. 2. **A:** PSMA enhancer sequence; grey arrows indicate positioning of primers used to create the deletion constructs. Numbering refers to Genbank accession number AF007544. **B:** Pictorial representation of the PSMA enhancer region of the deletion constructs.

moter. All results were normalized for transfection efficiency, and the mean and standard deviation of the experiments in triplicate were expressed as a percentage of the pGL3-control (SV-40 promoter and enhancer). All the enhancer deletions were able to drive prostate-specific expression of luciferase in both orientations, although with varying amounts of activity (see Fig. 3). The most active constructs were the pGL3-B-PSMA1648 and pGL3-B-PSMA1290 plasmids, which in the antisense orientation showed approximately 20-fold expression over the pGL3-control vector, and 200-fold over the PSMA promoter alone in C4-2 cells. Conversely, when the same constructs were transfected into breast cancer MCF-7 cells, the enhancer provided no additional activity over the PSMA-promoter alone. These results are consistent with those reported for the original characterization of the PSMA enhancer [13]. When the enhancer constructs were driven by the SV-40 promoter, the activity of each deletion was consistent with that seen in combination with the PSMA promoter, although the overall activity of the two best constructs, pGL3-B-PSMA1648 and pGL3-B-PSMA1290, was increased to about 25-fold of that of the pGL3 control vector in C4-2 cells. However, when the SV-40 promoter was tested with the enhancer deletions in MCF-7 cells, these same two constructs showed up to 20% of the activity of the pGL3-control. This suggests that there may be some elements of the PSMA promoter itself that contribute to prostate-specific expression. Given these results, we next took the pGL3-B-PSMA1648 plasmid and replaced the luciferase reporter gene with the *E. coli* CD gene to form pPSM/CD (sense or antisense, dependent on the orientation of the CD gene relative to the PSMA promoter).

Cytotoxicity/Proliferation Inhibition of 5-FU and 5-FC

In order to determine the sensitivity of each cell line to 5-FU, cytotoxicity/proliferation inhibition assays were performed (see Fig. 4 and Table I). The IC_{50} of 5-FU for all cell lines was similar, ranging between

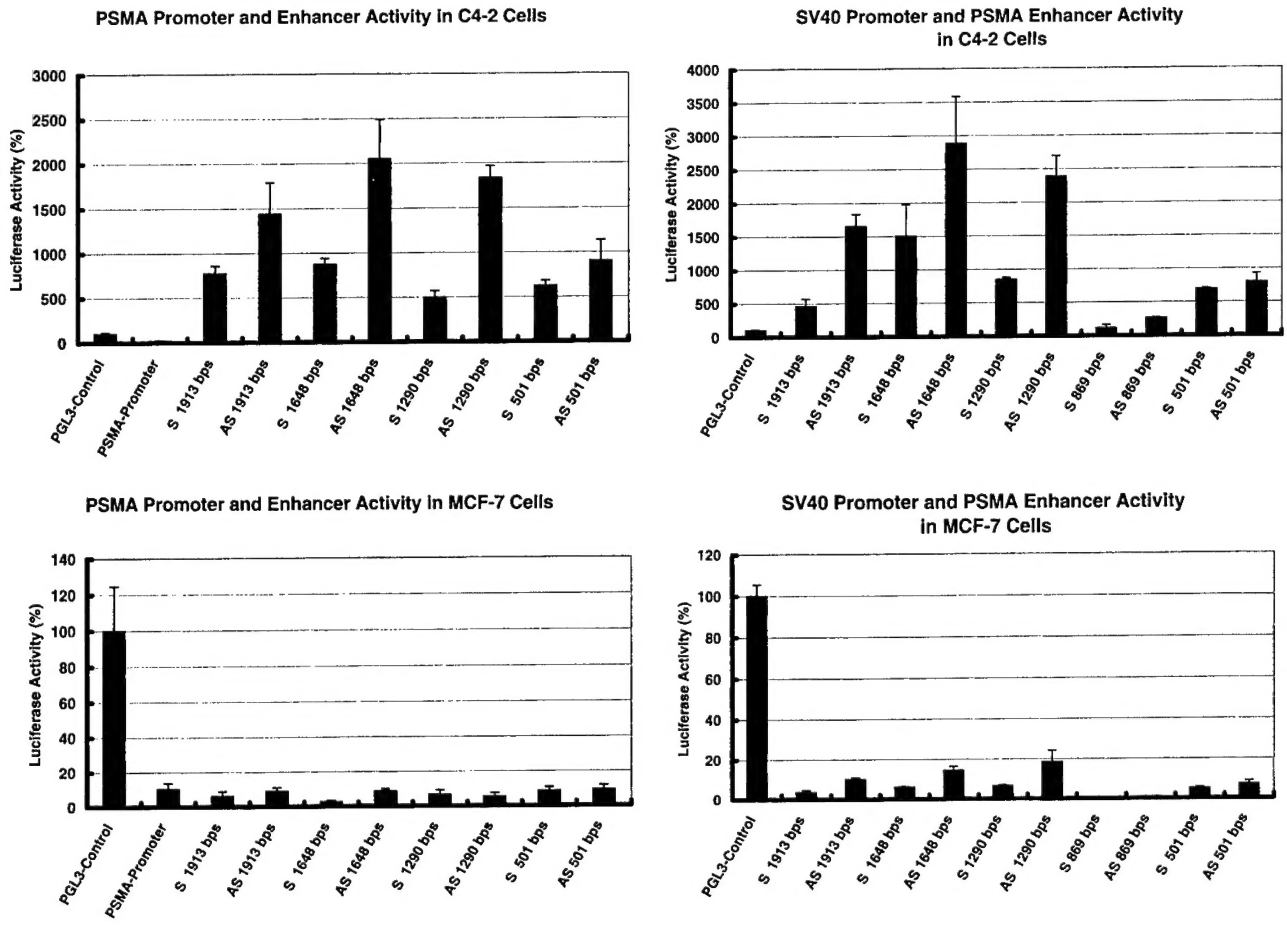

Fig. 3. Deletion analysis of the PSMA enhancer. Luciferase activity after normalization for transfection efficiency, expressed as percentage of positive control (pGL3-control), where the control was set at 100% activity. Enhancer deletions are indicated. S, enhancer in the sense orientation with respect to the promoter; AS, antisense orientation. Addition of PSMA enhancer to either the PSMA promoter or the SV40 promoter resulted in a significant (up to 300-fold) increase in reporter gene expression over the pGL3 control vector in the prostate cancer cell lines C4-2 and LNCaP, while minimal expression was seen in breast cancer cell line MCF-7. The most active deletions are the antisense 1,648-bp and 1,290-bp constructs, respectively.

1–10 μM according to several independent experiments. Compared to 5-FU, 5-FC is much less toxic for all cell lines. The IC_{50} of 5-FC for all cell lines was over 10 mM (more than 1,000 times that of 5-FU).

Cytotoxicity/Proliferation Inhibition in Transiently Transfected Cells

Transient transfection of LNCaP and C4-2 cells with pPSMA/CD-sense resulted in sensitization against 5-FC. In C4-2 cells, the 5-FC at 200, 300, and 400 μM inhibited cell growth to 67%, 39%, and 38%, respectively, compared to the control (nontransfected cells without 5-FC). The IC_{50} of 5-FC on transfected C4-2 was between 200–300 μM.

Compared to the IC_{50} of nontransfected cells (>10 mM), C4-2 was sensitized nearly 40-fold by transfection with pPSMA/CD-sense. It should be noted that the transfection efficiency of C4-2 cells was only 8% as determined by X-gal staining, and exposure to 5-FC was only 3 days. The enhanced cytotoxic effect of 5-FC was also noted in LNCaP cells when transfected with the pPSMA/CD-sense plasmid. However, the sensitization in LNCaP cells (IC_{50} 1–5 mM), was not as great as that in C4-2 cells. This could be because LNCaP cells are less sensitive to 5-FU treatment (see Table I), probably due to genetic differences between the two cell lines. Another cause could be that the recovery period of LNCaP after transfection is much longer than that of C4-2. Therefore, there is less chance of LNCaP cells incorporating 5-FU as their cell cycle is longer. In H157, HCT8, and MCF-7 cells, there was no significant difference in cell numbers between those transfected with pPSMA/CD sense and antisense constructs, demonstrating the specificity of the PSMA promoter/enhancer construct for prostate cells.

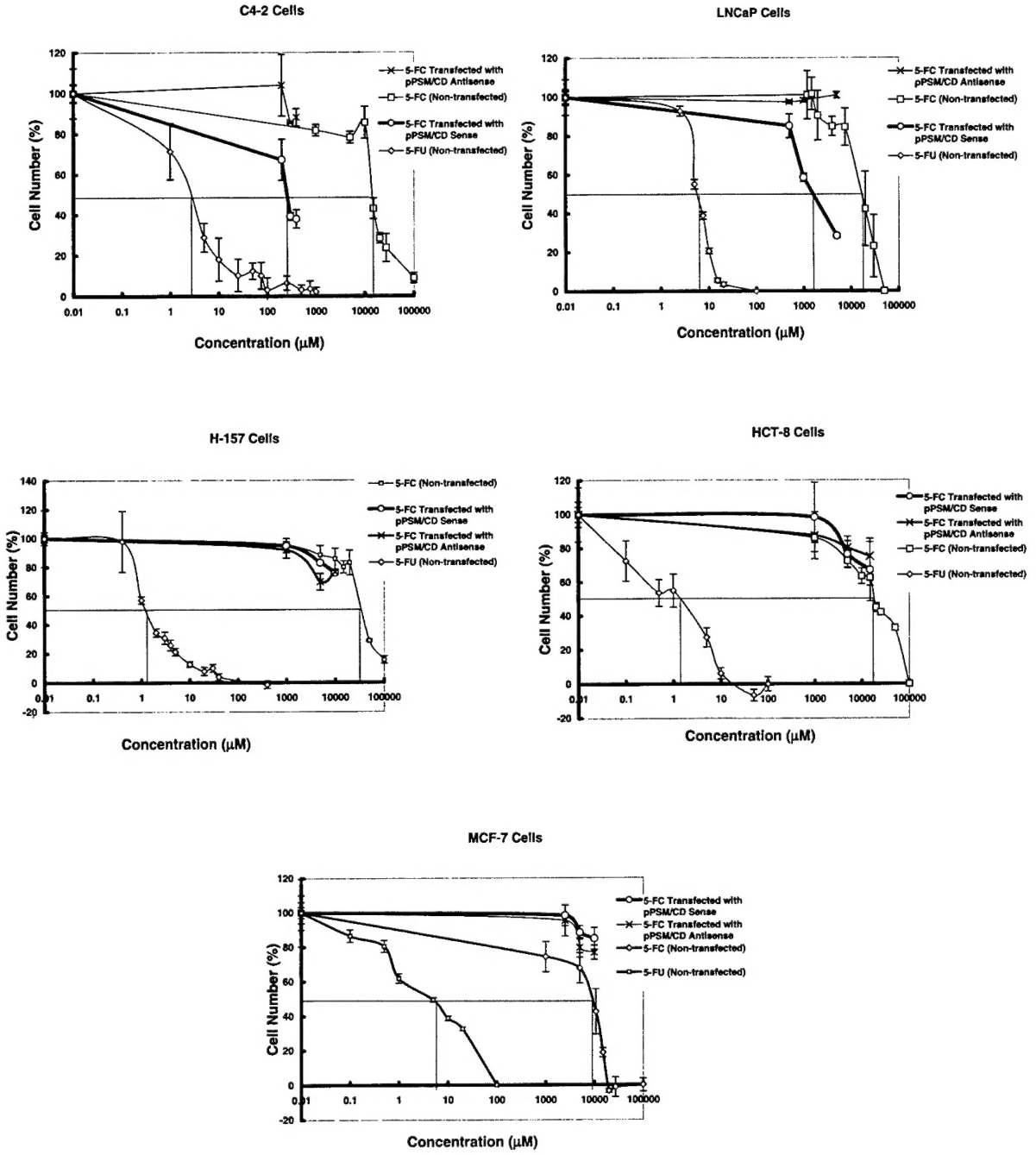

Fig. 4. Determination of IC_{50} of 5-fluorocytosine (5-FC) and 5-fluorouracil (5-FU) in nontransfected cells, and cells transfected with both sense and antisense cytosine deaminase, driven by the PSMA promoter and the 1,648-bp enhancer from the PSMA gene. The vertical line dropped from the 50% horizontal indicates the IC_{50}. It can be seen that transfection with the sense cytosine deaminase construct sensitizes LNCaP and C4-2 cells significantly to 5-FC treatment, while the nonprostatic cell lines H-157, HCT-8, and MCF-7 are not sensitized to 5-FC, demonstrating the specificity of this approach for prostate cancer.

Cytosine Deaminase Enzymatic Activity in Transfected Cells

The cytosine deaminase activity in C4-2 cell lysate was dependent on transfection efficiency. When 5% and 1% of C4-2 and 5% of LNCaP cells in a 100-mm dish were transfected with pPSM/CD-sense plasmid, the lysate showed an enzymatic activity of 2.4, 1.6, and 0.7 nmol/min/mg protein, respectively (data not shown). The enzymatic activity in the lysate of cells transfected with pPSM/CD-antisense plasmid, as well as H157 transfected with the pPSM/CD sense construct, did not show any detectable enzymatic activity of cytosine deaminase.

TABLE I. Comparison of Sensitivity to 5-Fluorouracil (5-FU) and 5-Fluorocytosine (5-FC) in Each Cell Line*

Cell line	Tissue origin	PSMA expression	IC_{50} 5-FU (μM)	IC_{50} 5-FC parental (μM)	IC_{50} 5-FC PSMA/CD (μM)	Ratio IC_{50} of 5-FC (parental)/ PSMA/CD)	Transfection efficiency (%)
C4-2	Prostate	+	1–5	15,000	200–300	>50	8
LNCaP	Prostate	+	1–10	15,000	1,000–2,000	10	5
H-157	Lung	–	1–10	>20,000	>10000	<2	10
HCT-8	Colon	–	1–5	20,000	>15000	<2	6
MCF-7	Breast	–	1–5	10,000	>10000	<2	15

*The inhibitory concentration 50% (IC_{50}) of 5-FU and 5-FC in all cell lines was very similar. However, when cells were transfected with the pPSMA/CD-sense plasmid, C4-2 and LNCaP cells were sensitized to 5-FC, 50- and 10-fold more, respectively, than the parental nontransfected cells, whereas the other nonprostatic cells were not significantly sensitized. Note that sensitization was achieved with only 8% transfection efficiency in C4-2 cells.

DISCUSSION

Gene therapy is in the process of becoming an acceptable treatment method for patients for whom there is no other effective treatment. Control of drug activation using prodrug therapy in combination with gene therapy is theoretically a way of staging a maximal attack on the targeted tumor, while controlling cytotoxicity to noncancerous tissue. To date, the two promoter/enhancers most often considered for use in prostate-specific therapy have been those of the rat probasin gene, and the human PSA gene. Both of these promoter/enhancers are positively regulated by androgens, and therefore would be of little use to patients who are undergoing androgen ablative therapy [15,16]. However, the PSMA gene is upregulated in the absence of androgens and is highly expressed in virtually all tumors and their metastases examined so far. As such, we chose to use regulatory elements from the PSMA gene to develop a construct that could be used to control expression of a "suicide gene" in a gene therapy approach against this disease. We recently cloned the promoter of the PSMA gene, which is able by itself to drive prostate-specific expression of a reporter gene; albeit at relatively low levels [11]. More recently, we isolated a prostate-specific enhancer from the third intron of the gene, which is upregulated in the absence of androgens [13]. Addition of the full-length enhancer to the PSMA promoter in a reporter construct results in approximately a 100-fold increase in expression. These data demonstrate that the enhancer retains full activity when positioned downstream of the gene; previous results were obtained with the enhancer upstream of the promoter [13]. As the full-length enhancer had not been tested in C4-2 cells, we initially compared its ability to drive the GFP reporter gene in a number of cell lines. While the enhancer clearly shows a significant increase in activity over the PSMA promoter alone in LNCaP and C4-2 cells, we were surprised to see more activity of the PSMA promoter alone in PC-3 cells than in either LNCaP or C4-2. PC-3 cells do not express PSMA; however, expression of the PSMA promoter would suggest that while the transcription factors necessary to drive the promoter's expression are available in PC-3 cells, there is some kind of defect in the PSMA promoter in these cells. Such a defect might be due to homozygous deletion or methylation of the PC-3 PSMA promoter. In addition, the enhancer did not seem to significantly increase expression over the promoter alone in PC-3 cells. This may be due to a number of reasons, e.g., the factors involved in upregulation via the enhancer are not present in PC-3 cells, and fluorescence microscopy is less quantitative than luciferase assays.

Interestingly, analysis of the deletion constructs revealed that there appear to be at least two distinct positive-regulatory regions within the enhancer region, as the smallest construct (501 bp) still exhibited more than a 50-fold increase in expression over the promoter alone. However, the most active constructs were clearly the 1,648-bp and 1,290-bp fragments, which showed a 175- to more than 200-fold increase in expression over the PSMA promoter alone. As this is more than the entire enhancer, it would suggest that there may be a negative regulator between nt 1648 and 1913 of the originally defined enhancer region. The identity of either of the positively or negatively regulating elements is unclear, as there are no consensus enhancer elements present in the PSMA enhancer sequence. There also appeared to be an effect of the orientation of the enhancer with respect to the promoter. While all the constructs showed significantly increased activity over both the SV-40 control and the PSMA-promoter alone, the constructs with the enhancer in the antisense orientation with respect to

their orientation in the native PSMA gene seemed to have significantly more activity, and the reason for this is unknown.

We tested the most active PSMA-promoter/enhancer/CD gene construct in vitro using a transient transfection approach, because it probably most resembles the principal problem with genetic therapy at the present time, i.e., delivery of the vector to the target tissue. One advantage of the cytosine deaminase/5-FC therapy approach is the bystander effect, in which cells close to the actual cell producing the engineered enzyme (in this case CD) are affected by the converted prodrug and destroyed. All results in this study were normalized for transfection efficiency, so that a logical comparison could be made between the different cell lines. However, even with the average transfection efficiency of C4-2 cells of around 8%, significant conversion of 5-FC to 5-FU and subsequent cell death were seen. Transfection with the suicide construct sensitized C4-2 cells to 5-FC nearly 40-fold, with an IC_{50} of between 200–300 μM. The usual oral dose of 5-FC for fungal treatment in humans is 37.5 mg per kg body weight every 6 hr, which results in peak serum concentrations of 540–620 μM in adults. The specificity of this method was shown by the lack of sensitization to 5-FC by transfection in the breast-, colon-, and lung-derived cell lines. However, it still remains to be determined if this enhancer is active in other tissues in vivo where PSMA is reportedly expressed. Other enhancers could be responsible for this nonprostatic expression, or the expression might be due to activation of another gene such as the PSMA-like gene. Furthermore, it will be important to determine if this PSMA enhancer is responsible for the expression of PSMA-protein seen in tumor neovasculature [10], as this would provide a much wider use for gene therapy constructs utilizing the PSMA regulatory control regions described here. We are attempting to answer both of these questions, in part by generating a transgenic mouse model carrying the human PSMA promoter and full-length enhancer-driving expression of green fluorescent protein.

CONCLUSIONS

We have identified the PSMA promoter and enhancer regulatory regions responsible for the strong expression of PSMA in prostate tumor cells such as LNCaP and C4-2. Furthermore, we have demonstrated that this PSMA-promoter/enhancer construct is capable of selectively driving expression of therapeutic genes, and we are exploring which gene or genes would be most useful along with ways to increase the effectiveness of vector delivery to the tumor or metastatic site. Suicide gene therapy for prostate cancer using the PSMA promoter and enhancer holds promise for the treatment of prostate cancer.

REFERENCES

1. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. CA Cancer J Clin 2000;50:7–33.
2. Danielsen S, Kilstrup M, Barilla K, Jochimsen B, Neuhard J. Characterization of the *Escherichia coli* codBA operon encoding cytosine permease and cytosine deaminase. Mol Microbiol 1992; 6:1335–1344.
3. Richards CA, Austin EA, Huber BE. Transcriptional regulatory sequences of carcinoembryonic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. Hum Gene Ther 1995;6:881–893.
4. Horoszewicz JS, Kawinski E, Murphy GP. Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. Anticancer Res 1987;7:927–935.
5. Israeli RS, Powell CT, Fair WR, Heston WD. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. Cancer Res 1993;53:227–230.
6. Israeli RS, Powell CT, Corr JG, Fair WR, Heston WD. Expression of the prostate-specific membrane antigen. Cancer Res 1994;54: 1807–1811.
7. Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C. Prostate-specific membrane antigen expression in normal and malignant human tissues. Clin Cancer Res 1997;3:81–85.
8. Liu H, Moy P, Kim S, Xia Y, Rajasekaran A, Navarro V, Knudsen B, Bander NH. Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. Cancer Res 1997;57:3629–3634.
9. Chang SS, Reuter VE, Heston WD, Bander NH, Grauer LS, Gaudin PB. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. Cancer Res 1999;59:3192–3198.
10. Chang SS, O'Keefe DS, Bacich DJ, Reuter VE, Heston WD, Gaudin PB. Prostate-specific membrane antigen is produced in tumor-associated neovasculature. Clin Cancer Res 1999;5:2674–2681.
11. O'Keefe DS, Su SL, Bacich DJ, Horiguchi Y, Luo Y, Powell CT, Zandvliet D, Russell PJ, Molloy PL, Nowak NJ, Shows TB, Mullins C, Vonder Haar RA, Fair WR, Heston WD. Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. Biochim Biophys Acta 1998;1443:113–127.
12. Uchida A, O'Keefe DS, Bacich DJ, Watt F, Molloy PL, Heston WDW. Prostate-specific suicide gene therapy using the newly discovered prostate-specific membrane antigen (PSMA) enhancer. Proc Am Assoc Cancer Res 2000;41:380.
13. Watt FB, Brookes DE, Ho T, Martorana A, Kingsley E, O'Keefe DS, Russell PJ, Heston WDW, Molloy PL. A tissue-specific enhancer of the prostate-specific membrane antigen gene. in press.
14. Nishiyama T, Kawamura Y, Kawamoto K, Matsumura H, Yamamoto N, Ito T, Ohyama A, Katsuragi T, Sakai T. Antineoplastic effects in rats of 5-fluorocytosine in combination with cytosine deaminase capsules. Cancer Res 1985;45:1753–1761.
15. Pang S, Taneja S, Dardashti K, Cohan P, Kaboo R, Sokoloff M, Tso CL, Dekernion JB, Belldegrun AS. Prostate tissue specificity of the prostate-specific antigen promoter isolated from a patient with prostate cancer. Hum Gene Ther 1995;6:1417–1426.
16. Brookes DE, Zandvliet D, Watt F, Russell PJ, Molloy PL. Relative activity and specificity of promoters from prostate-expressed genes. Prostate 1998;35:18–26.